5   ANTISENSE MODULATION OF SUPEROXIDE DISMUTASE 1, SOLUBLE
EXPRESSION

10   **RELATED APPLICATIONS**

This application is a continuation-in-part of U.S.
Application Serial No. 10/633,843, filed August 4, 2003,
which is a continuation of U.S. Application Serial No.
09/888,360, filed June 21, 2001.   The entire contents of
15   these priority documents are incorporated herein by
reference.

FIELD OF THE INVENTION

The present invention provides compositions and
20   methods for modulating the expression of superoxide
dismutase 1, soluble.   In particular, this invention
relates to compounds, particularly oligonucleotides,
specifically hybridizable with nucleic acids encoding
superoxide dismutase 1, soluble.   Such compounds have been
25   shown to modulate the expression of superoxide dismutase 1,
soluble.

BACKGROUND OF THE INVENTION

The superoxide anion (O_2^-) is a potentially harmful
30   cellular by-product produced primarily by errors of
oxidative phosphorylation in mitochondria (Cleveland and
Liu, *Nat. Med.*, **2000**, *6*, 1320-1321). Some of the targets
for oxidation by superoxide in biological systems include
the iron-sulfur dehydratases, aconitase and fumarases.
35   Release of Fe(II) from these superoxide-inactivated enzymes

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results in Fenton-type production of hydroxyl radicals which are capable of attacking virtually any cellular target, most notably DNA (Fridovich, *Annu. Rev. Biochem.*, **1995**, *64*, 97-112).

5   The enzymes known as the superoxide dismutases (SODs) provide defense against oxidative damage of biomolecules by catalyzing the dismutation of superoxide to hydrogen peroxide (H_2O_2) (Fridovich, *Annu. Rev. Biochem.*, **1995**, *64*, 97-112). Two major classes of superoxide dismutases exist.

10   One consists of a group of enzymes with active sites containing copper and zinc while the other class has either manganese or iron at the active site (Fridovich, *Annu. Rev. Biochem.*, **1995**, *64*, 97-112).

     The soluble superoxide dismutase 1 enzyme (also known

15   as SOD1 and Cu/Zn superoxide dismutase) contains a zinc- and copper-type active site (Fridovich, *Annu. Rev. Biochem.*, **1995**, *64*, 97-112). Lee et al. reported the molecular cloning and high-level expression of human cytoplasmic superoxide dismutase gene in *E. coli* in 1990

20   (Lee et al., *Misaengmul Hakhoechi*, **1990**, *28*, 91-97).

     Mutations in the superoxide dismutase 1 gene are associated with a dominantly-inherited form of amyotrophic lateral sclerosis (ALS, also known as Lou Gehrig's disease) a disorder characterized by a selective degeneration of

25   upper and lower motor neurons (Cleveland and Liu, *Nat. Med.*, **2000**, *6*, 1320-1321). The deleterious effects of various mutations on superoxide dismutase 1 are most likely mediated through a gain of toxic function rather than a loss of superoxide dismutase 1 activity, as the complete

30   absence of superoxide dismutase 1 in mice neither diminishes life nor provokes overt disease (Al-Chalabi and Leigh, *Curr. Opin. Neurol.*, **2000**, *13*, 397-405; Alisky and Davidson, *Hum. Gene Ther.*, **2000**, *11*, 2315-2329). According to Cleveland and Liu, there are two models for

mutant superoxide dismutase 1 toxicity (Cleveland and Liu, *Nat. Med.*, **2000**, *6*, 1320-1321). The ''oxidative hypothesis'' ascribes toxicity to binding of aberrant substrates such as peroxynitrite or hydrogen peroxide which gain access to the

5   catalytic copper ion through mutation-dependent loosening of the native superoxide dismutase 1 protein conformation (Cleveland and Liu, *Nat. Med.*, **2000**, *6*, 1320-1321). A second possible mechanism for mutant superoxide dismutase 1 toxicity involves the misfolding and aggregation of mutant

10   superoxide dismutase 1 proteins (Cleveland and Liu, *Nat. Med.*, **2000**, *6*, 1320-1321). The idea that aggregates contribute to ALS has received major support from the observation that murine models of superoxide dismutase 1 mutant-mediated disease feature prominent intracellular

15   inclusions in motor neurons and, in some cases, in the astrocytes surrounding them as well (Bruijn et al., *Science,* **1998**, *281*, 1851-1854). Furthermore, Brujin et al. also demonstrate that neither elimination nor elevation of wild-type superoxide dismutase 1 was found to affect

20   disease induced by mutant superoxide dismutase 1 in mice (Bruijn et al., *Science,* **1998**, *281*, 1851-1854).

The superoxide dismutase 1 gene is localized to chromosome 21q22.1 and has been found to be overexpressed in the brains of patients with Down syndrome, possibly as a

25   reflection of the trisomic state of chromosome 21 (Gulesserian et al., *J. Investig. Med.,* **2001**, *49*, 41-46).

Studies of transgenic mice carrying a mutant human superoxide dismutase 1 gene have been used to evaluate potential therapies for ALS and one such study has

30   indicated that creatine produced a dose-dependent improvement in motor performance and extended survival in mice containing the glycine 93 to alanine mutation (Klivenyi et al., *Nat. Med.,* **1999**, *5*, 347-350). Although creatine is currently suggested as a dietary supplement for

35   patients with ALS, the protective effect of creatine in

humans has yet to be confirmed (Rowland, *J. Neurol. Sci.*, **2000**, *180*, 2-6).

Additional transgenic mice studies have led to the finding that oxidative reactions triggered by superoxide dismutase 1 mutants result in inactivation of the glial glutamate transporter (Human GLUT1) which in turn, causes neuronal degeneration (Trotti et al., *Nat. Neurosci.*, **1999**, *2*, 427-433).

Inhibition of superoxide dismutase 1 through copper chelation or zinc supplementation extends the life of mice that overexpress a mutant form superoxide dismutase by 1 to 2 months (Hottinger et al., *Eur. J. Neurosci.*, **1997**, *9*, 1548-1551). As reviewed by Alisky and Davidson, a number of pharmacological agents have been used to inhibit the toxicity of superoxide dismutase 1 mutants in the transgenic mouse model for human ALS, including: vitamin E, riluzole, gabapentin, caspase inhibitors, nitric oxide synthase inhibitors, glutamate receptor inhibitors and glutathione (Alisky and Davidson, *Hum. Gene Ther.*, **2000**, *11*, 2315-2329). In addition, investigational gene therapy for ALS has included overexpression of a number of genes which provide protection from superoxide dismutase 1 mutant toxicity (Alisky and Davidson, *Hum. Gene Ther.*, **2000**, *11*, 2315-2329).

Two abnormal superoxide dismutase 1 mRNAs, exon 2-skipping and exon 2 and 3-skipping species, were identified from occipital brain tissue of ALS patients carrying no mutations in the superoxide dismutase 1 gene (Kawata et al., *NeuroReport*, **2000**, *11*, 2649-2653).

Disclosed and claimed in PCT publication WO 94/19493 are oligonucleotide sequences encoding SOD1 and generally claimed is the use of an antisense DNA homolog of a gene encoding SOD1 in either mutant and wild-type forms in the preparation of a medicament for treating a patient with a disease (Brown et al., **1994**).

The expression of superoxide dismutase 1 in PC12 rat pheochromocytoma neuronal cells was inhibited by either of two 1-mer antisense oligonucleotides targeting rat superoxide dismutase 1 nucleotides 54-74 and 497-517,

5   leading to cellular apoptosis. The progression of cellular death was reversed by treatment with antioxidants (Troy and Shelanski, *Proc. Natl. Acad. Sci. U. S. A.*, **1994**, *91*, 6384-6387).

The method of delivery of the previously described

10   oligonucleotides to the rat PC12 cells (Troy and Shelanski, *Proc. Natl. Acad. Sci. U. S. A.*, **1994**, *91*, 6384-6387) was subsequently improved by linking the oligonucleotides to a vector peptide via a disulfide bond (Troy et al., *J. Neurosci.*, **1996**, *16*, 253-261).

15   Induction of apoptosis was also seen in studies using a 30-mer phosphorothioate oligonucleotide targeting the start codon of superoxide dismutase 1 in rat spinal cord cultures *in vitro* (Rothstein et al., *Proc. Natl. Acad. Sci. U. S. A.*, **1994**, *91*, 4155-4159).

20   Mutations of the superoxide dismutase 1 gene have been unambiguously implicated in ALS. However, investigational therapies involving inhibition of these mutants have yet to be tested as therapeutic protocols. Furthermore, evidence suggests that inhibition of the wild-type superoxide

25   dismutase gene is not deleterious to organisms (Bruijn et al., *Science*, **1998**, *281*, 1851-1854). Consequently there remains a long felt need for agents capable of effectively and selectively inhibiting superoxide dismutase 1 function.

Antisense technology is emerging as an effective means

30   for reducing the expression of specific gene products and may therefore prove to be uniquely useful in a number of therapeutic, diagnostic, and research applications for the modulation of superoxide dismutase 1 expression.

The present invention provides compositions and

35   methods for modulating human superoxide dismutase 1

expression, including modulation of alternatively spliced forms of superoxide dismutase 1.

SUMMARY OF THE INVENTION

5   The present invention is directed to compounds, particularly antisense oligonucleotides, which are targeted to a nucleic acid encoding superoxide dismutase 1, soluble, and which modulate the expression of superoxide dismutase 1, soluble. Pharmaceutical and other compositions

10   comprising the compounds of the invention are also provided. Further provided are methods of modulating the expression of superoxide dismutase 1, soluble in cells or tissues comprising contacting said cells or tissues with one or more of the antisense compounds or compositions of

15   the invention. Further provided are methods of treating an animal, particularly a human, suspected of having or being prone to a disease or condition associated with expression of superoxide dismutase 1, soluble by administering a therapeutically or prophylactically effective amount of one

20   or more of the antisense compounds or compositions of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

        Figure 1 is a bar graph showing inhibition of

25   superoxide dismutase 1, soluble (SOD-1) mRNA in the rat cervical spinal cord after intraventricular administration of ISIS 146192, a SOD-1 antisense oligonucleotide. Sod=SOD-1. PTEN was used to show that ISIS 146192 is specific for SOD-1 and does not decrease levels of PTEN

30   mRNA. mRNA levels were normalized to ribogreen.

        Figure 2 is a bar graph showing inhibition of SOD-1 mRNA in the rat cervical spinal cord after intraventricular administration of ISIS 146192. mRNA levels were normalized to β-actin.

35   Figure 3 is a bar graph showing inhibition of SOD-1 mRNA in the right temporal parietal section of the rat

brain after intraventricular administration of ISIS 146192.
mRNA levels were normalized to ribogreen.

   Figure 4 is a bar graph showing inhibition of SOD-1
mRNA in the right temporal parietal section of the rat
5 brain after intraventricular administration of ISIS 146192.
mRNA levels were normalized to β-actin.

DETAILED DESCRIPTION OF THE INVENTION

   The present invention employs oligomeric compounds,
10 particularly antisense oligonucleotides, for use in
modulating the function of nucleic acid molecules encoding
superoxide dismutase 1, soluble, ultimately modulating the
amount of superoxide dismutase 1, soluble produced. This
is accomplished by providing antisense compounds which
15 specifically hybridize with one or more nucleic acids
encoding superoxide dismutase 1, soluble. As used herein,
the terms "target nucleic acid" and "nucleic acid encoding
superoxide dismutase 1, soluble" encompass DNA encoding
superoxide dismutase 1, soluble, RNA (including pre-mRNA
20 and mRNA) transcribed from such DNA, and also cDNA derived
from such RNA. The specific hybridization of an oligomeric
compound with its target nucleic acid interferes with the
normal function of the nucleic acid. This modulation of
function of a target nucleic acid by compounds which
25 specifically hybridize to it is generally referred to as
"antisense". The functions of DNA to be interfered with
include replication and transcription. The functions of
RNA to be interfered with include all vital functions such
as, for example, translocation of the RNA to the site of
30 protein translation, translation of protein from the RNA,
splicing of the RNA to yield one or more mRNA species, and
catalytic activity which may be engaged in or facilitated
by the RNA. The overall effect of such interference with
target nucleic acid function is modulation of the
35 expression of superoxide dismutase 1, soluble. In the
context of the present invention, "modulation" means either
an increase (stimulation) or a decrease (inhibition) in the

expression of a gene. In the context of the present
invention, inhibition is the preferred form of modulation
of gene expression and mRNA is a preferred target.

5   It is preferred to target specific nucleic acids for
antisense. "Targeting" an antisense compound to a
particular nucleic acid, in the context of this invention,
is a multistep process. The process usually begins with
the identification of a nucleic acid sequence whose
function is to be modulated. This may be, for example, a
10   cellular gene (or mRNA transcribed from the gene) whose
expression is associated with a particular disorder or
disease state, or a nucleic acid molecule from an
infectious agent. In the present invention, the target is
a nucleic acid molecule encoding superoxide dismutase 1,
15   soluble. The targeting process also includes determination
of a site or sites within this gene for the antisense
interaction to occur such that the desired effect, e.g.,
detection or modulation of expression of the protein, will
result. Within the context of the present invention, a
20   preferred intragenic site is the region encompassing the
translation initiation or termination codon of the open
reading frame (ORF) of the gene. Since, as is known in the
art, the translation initiation codon is typically 5'-AUG
(in transcribed mRNA molecules; 5'-ATG in the corresponding
25   DNA molecule), the translation initiation codon is also
referred to as the "AUG codon," the "start codon" or the
"AUG start codon". A minority of genes have a translation
initiation codon having the RNA sequence 5'-GUG, 5'-UUG or
5'-CUG, and 5'-AUA, 5'-ACG and 5'-CUG have been shown to
30   function in vivo. Thus, the terms "translation initiation
codon" and "start codon" can encompass many codon
sequences, even though the initiator amino acid in each
instance is typically methionine (in eukaryotes) or
formylmethionine (in prokaryotes). It is also known in the
35   art that eukaryotic and prokaryotic genes may have two or
more alternative start codons, any one of which may be
preferentially utilized for translation initiation in a

particular cell type or tissue, or under a particular set
of conditions. In the context of the invention, "start
codon" and "translation initiation codon" refer to the
codon or codons that are used in vivo to initiate
5   translation of an mRNA molecule transcribed from a gene
encoding superoxide dismutase 1, soluble, regardless of the
sequence(s) of such codons.

It is also known in the art that a translation
termination codon (or "stop codon") of a gene may have one
10   of three sequences, i.e., 5'-UAA, 5'-UAG and 5'-UGA (the
corresponding DNA sequences are 5'-TAA, 5'-TAG and 5'-TGA,
respectively). The terms "start codon region" and
"translation initiation codon region" refer to a portion of
such an mRNA or gene that encompasses from about 25 to
15   about 50 contiguous nucleotides in either direction (i.e.,
5' or 3') from a translation initiation codon. Similarly,
the terms "stop codon region" and "translation termination
codon region" refer to a portion of such an mRNA or gene
that encompasses from about 25 to about 50 contiguous
20   nucleotides in either direction (i.e., 5' or 3') from a
translation termination codon.

The open reading frame (ORF) or "coding region," which
is known in the art to refer to the region between the
translation initiation codon and the translation
25   termination codon, is also a region which may be targeted
effectively. Other target regions include the 5'
untranslated region (5'UTR), known in the art to refer to
the portion of an mRNA in the 5' direction from the
translation initiation codon, and thus including
30   nucleotides between the 5' cap site and the translation
initiation codon of an mRNA or corresponding nucleotides on
the gene, and the 3' untranslated region (3'UTR), known in
the art to refer to the portion of an mRNA in the 3'
direction from the translation termination codon, and thus
35   including nucleotides between the translation termination
codon and 3' end of an mRNA or corresponding nucleotides on
the gene. The 5' cap of an mRNA comprises an N7-methylated

guanosine residue joined to the 5'-most residue of the mRNA via a 5'-5' triphosphate linkage. The 5' cap region of an mRNA is considered to include the 5' cap structure itself as well as the first 50 nucleotides adjacent to the cap.

5 The 5' cap region may also be a preferred target region.

Although some eukaryotic mRNA transcripts are directly translated, many contain one or more regions, known as "introns," which are excised from a transcript before it is translated. The remaining (and therefore translated)

10 regions are known as "exons" and are spliced together to form a continuous mRNA sequence. mRNA splice sites, i.e., intron-exon junctions, may also be preferred target regions, and are particularly useful in situations where aberrant splicing is implicated in disease, or where an

15 overproduction of a particular mRNA splice product is implicated in disease. Aberrant fusion junctions due to rearrangements or deletions are also preferred targets. It has also been found that introns can also be effective, and therefore preferred, target regions for antisense compounds

20 targeted, for example, to DNA or pre-mRNA.

Once one or more target sites have been identified, oligonucleotides are chosen which are sufficiently complementary to the target, i.e., hybridize sufficiently well and with sufficient specificity, to give the desired

25 effect.

In the context of this invention, "hybridization" means hydrogen bonding, which may be Watson-Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding, between complementary nucleoside or nucleotide bases. For example,

30 adenine and thymine are complementary nucleobases which pair through the formation of hydrogen bonds. "Complementary," as used herein, refers to the capacity for precise pairing between two nucleotides. For example, if a nucleotide at a certain position of an oligonucleotide is

35 capable of hydrogen bonding with a nucleotide at the same position of a DNA or RNA molecule, then the oligonucleotide and the DNA or RNA are considered to be complementary to

each other at that position. The oligonucleotide and the
DNA or RNA are complementary to each other when a
sufficient number of corresponding positions in each
molecule are occupied by nucleotides which can hydrogen
5   bond with each other. Thus, "specifically hybridizable"
and "complementary" are terms which are used to indicate a
sufficient degree of complementarity or precise pairing
such that stable and specific binding occurs between the
oligonucleotide and the DNA or RNA target. It is
10 understood in the art that the sequence of an antisense
compound need not be 100% complementary to that of its
target nucleic acid to be specifically hybridizable. An
antisense compound is specifically hybridizable when
binding of the compound to the target DNA or RNA molecule
15 interferes with the normal function of the target DNA or
RNA to cause a loss of utility, and there is a sufficient
degree of complementarity to avoid non-specific binding of
the antisense compound to non-target sequences under
conditions in which specific binding is desired, i.e.,
20 under physiological conditions in the case of in vivo
assays or therapeutic treatment, and in the case of in
vitro assays, under conditions in which the assays are
performed.

Antisense and other compounds of the invention which
25 hybridize to the target and inhibit expression of the
target are identified through experimentation, and the
sequences of these compounds are hereinbelow identified as
preferred embodiments of the invention. The target sites to
which these preferred sequences are complementary are
30 hereinbelow referred to as "active sites" and are therefore
preferred sites for targeting. Therefore another embodiment
of the invention encompasses compounds which hybridize to
these active sites.

Antisense compounds are commonly used as research
35 reagents and diagnostics. For example, antisense
oligonucleotides, which are able to inhibit gene expression
with exquisite specificity, are often used by those of

ordinary skill to elucidate the function of particular genes. Antisense compounds are also used, for example, to distinguish between functions of various members of a biological pathway. Antisense modulation has, therefore,

5   been harnessed for research use.

For use in kits and diagnostics, the antisense compounds of the present invention, either alone or in combination with other antisense compounds or therapeutics, can be used as tools in differential and/or combinatorial

10 analyses to elucidate expression patterns of a portion or the entire complement of genes expressed within cells and tissues.

Expression patterns within cells or tissues treated with one or more antisense compounds are compared to

15 control cells or tissues not treated with antisense compounds and the patterns produced are analyzed for differential levels of gene expression as they pertain, for example, to disease association, signaling pathway, cellular localization, expression level, size, structure or

20 function of the genes examined. These analyses can be performed on stimulated or unstimulated cells and in the presence or absence of other compounds which affect expression patterns.

Examples of methods of gene expression analysis known

25 in the art include DNA arrays or microarrays (Brazma and Vilo, *FEBS Lett.*, **2000,** 480, 17-24; Celis, et al., *FEBS Lett.*, **2000,** 480, 2-16), SAGE (serial analysis of gene expression)(Madden, et al., *Drug Discov. Today,* **2000,** 5, 415-425), READS (restriction enzyme amplification of

30 digested cDNAs) (Prashar and Weissman, *Methods Enzymol.*, **1999,** 303, 258-72), TOGA (total gene expression analysis) (Sutcliffe, et al., *Proc. Natl. Acad. Sci. U. S. A.,* **2000,** 97, 1976-81), protein arrays and proteomics (Celis, et al., *FEBS Lett.*, **2000,** 480, 2-16; Jungblut, et al.,

35 *Electrophoresis,* **1999,** 20, 2100-10), expressed sequence tag

(EST) sequencing (Celis, et al., *FEBS Lett.*, **2000**, 480, 2-
16; Larsson, et al., *J. Biotechnol.*, **2000**, 80, 143-57),
subtractive RNA fingerprinting (SuRF) (Fuchs, et al., *Anal.*
Biochem., **2000**, 286, 91-98; Larson, et al., *Cytometry,*
5   **2000**, 41, 203-208), subtractive cloning, differential
display (DD) (Jurecic and Belmont, *Curr. Opin. Microbiol.*,
2000, 3, 316-21), comparative genomic hybridization
(Carulli, et al., *J. Cell Biochem. Suppl.*, **1998**, 31, 286-
96), FISH (fluorescent in situ hybridization) techniques
10   (Going and Gusterson, *Eur. J. Cancer,* **1999**, 35, 1895-904)
and mass spectrometry methods (reviewed in (To, *Comb. Chem.*
High Throughput Screen, **2000**, 3, 235-41).

The specificity and sensitivity of antisense is also
harnessed by those of skill in the art for therapeutic
15   uses. Antisense oligonucleotides have been employed as
therapeutic moieties in the treatment of disease states in
animals and man. Antisense oligonucleotide drugs,
including ribozymes, have been safely and effectively
administered to humans and numerous clinical trials are
20   presently underway. It is thus established that
oligonucleotides can be useful therapeutic modalities that
can be configured to be useful in treatment regimes for
treatment of cells, tissues and animals, especially humans.

In the context of this invention, the term
25   "oligonucleotide" refers to an oligomer or polymer of
ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) or
mimetics thereof. This term includes oligonucleotides
composed of naturally-occurring nucleobases, sugars and
covalent internucleoside (backbone) linkages as well as
30   oligonucleotides having non-naturally-occurring portions
which function similarly. Such modified or substituted
oligonucleotides are often preferred over native forms
because of desirable properties such as, for example,
enhanced cellular uptake, enhanced affinity for nucleic
35

acid target and increased stability in the presence of nucleases.

   While antisense oligonucleotides are a preferred form of antisense compound, the present invention comprehends
5 other oligomeric antisense compounds, including but not limited to oligonucleotide mimetics such as are described below. The antisense compounds in accordance with this invention preferably comprise from about 8 to about 50 nucleobases (i.e. from about 8 to about 50 linked
10 nucleosides). Particularly preferred antisense compounds are antisense oligonucleotides, even more preferably those comprising from about 12 to about 30 nucleobases. Antisense compounds include ribozymes, external guide sequence (EGS) oligonucleotides (oligozymes), and other short catalytic
15 RNAs or catalytic oligonucleotides which hybridize to the target nucleic acid and modulate its expression.

   While the preferred form of antisense compound is a single-stranded antisense oligonucleotide, in many species the introduction of double-stranded structures, such as
20 double-stranded RNA (dsRNA) molecules, has been shown to induce potent and specific antisense-mediated reduction of the function of a gene or its associated gene products. This phenomenon occurs in both plants and animals and is believed to have an evolutionary connection to viral
25 defense and transposon silencing.

   The first evidence that dsRNA could lead to gene silencing in animals came in 1995 from work in the nematode, *Caenorhabditis elegans* (Guo and Kempheus, *Cell*, **1995**, 81, 611-620). Montgomery et al. have shown that the
30 primary interference effects of dsRNA are posttranscriptional (Montgomery et al., *Proc. Natl. Acad. Sci. USA*, **1998**, *95*, 15502-15507). The posttranscriptional antisense mechanism defined in *Caenorhabditis elegans* resulting from exposure to double-stranded RNA (dsRNA) has
35 since been designated RNA interference (RNAi). This term has been generalized to mean antisense-mediated gene

silencing involving the introduction of dsRNA leading to the sequence-specific reduction of endogenous targeted mRNA levels (Fire et al., *Nature*, **1998**, 391, 806-811). Recently, it has been shown that it is, in fact, the single-stranded

5   RNA oligomers of antisense polarity of the dsRNAs which are the potent inducers of RNAi (Tijsterman et al., *Science*, **2002**, *295*, 694-697). The use of these double stranded RNA molecules (short interfering RNA or siRNA) for targeting and inhibiting the expression of superoxide dismutase 1,

10   soluble mRNA is also contemplated. These double stranded RNA molecules target regions similar to those targeted by antisense oligocleotides and have similar effects. These double stranded RNA molecules are generally 19-21 base pairs in length, but may range between 8 and 50

15   nucleobases. The production of siRNA molecules is described in a general sense in the examples provided below, but it will be appreciated that any desired siRNA targeted to superoxide-1 dismutase, soluble may be synthesized by conventional oligonucleotide synthesis

20   techniques. Once the sequence of the antisense strand is known, the complementary sense strand is synthesized based on base pairing. The sense and antisense strands are then combined to form the siRNA.

25   **Oligomer and Monomer Modifications**

As is known in the art, a nucleoside is a base-sugar combination. The base portion of the nucleoside is normally a heterocyclic base. The two most common classes of such heterocyclic bases are the purines and the

30   pyrimidines. Nucleotides are nucleosides that further include a phosphate group covalently linked to the sugar portion of the nucleoside. For those nucleosides that include a pentofuranosyl sugar, the phosphate group can be linked to either the 2', 3' or 5' hydroxyl moiety of the

35   sugar. In forming oligonucleotides, the phosphate groups covalently link adjacent nucleosides to one another to form

a linear polymeric compound. In turn, the respective ends
of this linear polymeric compound can be further joined to
form a circular compound, however, linear compounds are
generally preferred. In addition, linear compounds may

5   have internal nucleobase complementarity and may therefore
fold in a manner as to produce a fully or partially double-
stranded compound. Within oligonucleotides, the phosphate
groups are commonly referred to as forming the
internucleoside linkage or in conjunction with the sugar

10 ring the backbone of the oligonucleotide. The normal
internucleoside linkage that makes up the backbone of RNA
and DNA is a 3' to 5' phosphodiester linkage.

Modified Internucleoside Linkages

15   Specific examples of preferred antisense oligomeric
compounds useful in this invention include oligonucleotides
containing modified e.g. non-naturally occurring
internucleoside linkages. As defined in this
specification, oligonucleotides having modified

20 internucleoside linkages include internucleoside linkages
that retain a phosphorus atom and internucleoside linkages
that do not have a phosphorus atom. For the purposes of
this specification, and as sometimes referenced in the art,
modified oligonucleotides that do not have a phosphorus

25 atom in their internucleoside backbone can also be
considered to be oligonucleosides.

In the *C. elegans* system, modification of the
internucleotide linkage (phosphorothioate) did not
significantly interfere with RNAi activity. Based on this

30 observation, it is suggested that certain preferred
oligomeric compounds of the invention can also have one or
more modified internucleoside linkages. A preferred
phosphorus containing modified internucleoside linkage is
the phosphorothioate internucleoside linkage.

35   Preferred modified oligonucleotide backbones
containing a phosphorus atom therein include, for example,
phosphorothioates, chiral phosphorothioates, phosphoro-

dithioates, phosphotriesters, aminoalkylphosphotriesters, methyl and other alkyl phosphonates including 3'-alkylene phosphonates, 5'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates including 3'-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, selenophosphates and borano-phosphates having normal 3'-5' linkages, 2'-5' linked analogs of these, and those having inverted polarity wherein one or more internucleotide linkages is a 3' to 3', 5' to 5' or 2' to 2' linkage. Preferred oligonucleotides having inverted polarity comprise a single 3' to 3' linkage at the 3'-most internucleotide linkage i.e. a single inverted nucleoside residue which may be abasic (the nucleobase is missing or has a hydroxyl group in place thereof). Various salts, mixed salts and free acid forms are also included.

Representative United States patents that teach the preparation of the above phosphorus-containing linkages include, but are not limited to, U.S.: 3,687,808; 4,469,863; 4,476,301; 5,023,243; 5,177,196; 5,188,897; 5,264,423; 5,276,019; 5,278,302; 5,286,717; 5,321,131; 5,399,676; 5,405,939; 5,453,496; 5,455,233; 5,466,677; 5,476,925; 5,519,126; 5,536,821; 5,541,306; 5,550,111; 5,563,253; 5,571,799; 5,587,361; 5,194,599; 5,565,555; 5,527,899; 5,721,218; 5,672,697 and 5,625,050, certain of which are commonly owned with this application, and each of which is herein incorporated by reference.

In more preferred embodiments of the invention, oligomeric compounds have one or more phosphorothioate and/or heteroatom internucleoside linkages, in particular -CH_2-NH-O-CH_2-, -CH_2-N(CH_3)-O-CH_2- [known as a methylene (methylimino) or MMI backbone], -CH_2-O-N(CH_3)-CH_2-, -CH_2-N(CH_3)-N(CH_3)-CH_2- and -O-N(CH_3)-CH_2-CH_2- [wherein the native phosphodiester internucleotide linkage is represented as -O-P(=O)(OH)-O-CH_2-]. The MMI type internucleoside linkages are disclosed in the above referenced U.S. patent

5,489,677. Preferred amide internucleoside linkages are disclosed in the above referenced U.S. patent 5,602,240.

Preferred modified oligonucleotide backbones that do not include a phosphorus atom therein have backbones that are formed by short chain alkyl or cycloalkyl internucleoside linkages, mixed heteroatom and alkyl or cycloalkyl internucleoside linkages, or one or more short chain heteroatomic or heterocyclic internucleoside linkages. These include those having morpholino linkages (formed in part from the sugar portion of a nucleoside); siloxane backbones; sulfide, sulfoxide and sulfone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; riboacetyl backbones; alkene containing backbones; sulfamate backbones; methyleneimino and methylenehydrazino backbones; sulfonate and sulfonamide backbones; amide backbones; and others having mixed N, O, S and CH_2 component parts.

Representative United States patents that teach the preparation of the above oligonucleosides include, but are not limited to, U.S.: 5,034,506; 5,166,315; 5,185,444; 5,214,134; 5,216,141; 5,235,033; 5,264,562; 5,264,564; 5,405,938; 5,434,257; 5,466,677; 5,470,967; 5,489,677; 5,541,307; 5,561,225; 5,596,086; 5,602,240; 5,610,289; 5,602,240; 5,608,046; 5,610,289; 5,618,704; 5,623,070; 5,663,312; 5,633,360; 5,677,437; 5,792,608; 5,646,269 and 5,677,439, certain of which are commonly owned with this application, and each of which is herein incorporated by reference.

Oligomer Mimetics

Another preferred group of oligomeric compounds amenable to the present invention includes oligonucleotide mimetics. The term mimetic as it is applied to oligonucleotides is intended to include oligomeric compounds wherein only the furanose ring or both the furanose ring and the internucleotide linkage are replaced

with novel groups, replacement of only the furanose ring is also referred to in the art as being a sugar surrogate. The heterocyclic base moiety or a modified heterocyclic base moiety is maintained for hybridization with an

5   appropriate target nucleic acid. One such oligomeric compound, an oligonucleotide mimetic that has been shown to have excellent hybridization properties, is referred to as a peptide nucleic acid (PNA). In PNA oligomeric compounds, the sugar-backbone of an oligonucleotide is replaced with

10   an amide containing backbone, in particular an aminoethylglycine backbone. The nucleobases are retained and are bound directly or indirectly to aza nitrogen atoms of the amide portion of the backbone. Representative United States patents that teach the preparation of PNA

15   oligomeric compounds include, but are not limited to, U.S.: 5,539,082; 5,714,331; and 5,719,262, each of which is herein incorporated by reference. Further teaching of PNA oligomeric compounds can be found in Nielsen et al., Science, **1991**, 254, 1497-1500.

20   One oligonucleotide mimetic that has been reported to have excellent hybridization properties is peptide nucleic acids (PNA). The backbone in PNA compounds is two or more linked aminoethylglycine units which gives PNA an amide containing backbone. The heterocyclic base moieties are

25   bound directly or indirectly to aza nitrogen atoms of the amide portion of the backbone. Representative United States patents that teach the preparation of PNA compounds include, but are not limited to, U.S.: 5,539,082; 5,714,331; and 5,719,262, each of which is herein

30   incorporated by reference. Further teaching of PNA compounds can be found in Nielsen et al., Science, 1991, 254, 1497-1500.

         PNA has been modified to incorporate numerous modifications since the basic PNA structure was first

35   prepared. The basic structure is shown below:
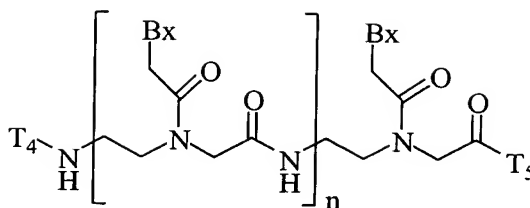
wherein

Bx is a heterocyclic base moiety;

T_4 is hydrogen, an amino protecting group, $-C(O)R_5$,
substituted or unsubstituted C_1-C_{10} alkyl, substituted or
unsubstituted C_2-C_{10} alkenyl, substituted or unsubstituted
C_2-C_{10} alkynyl, alkylsulfonyl, arylsulfonyl, a chemical
functional group, a reporter group, a conjugate group, a D
or L α-amino acid linked via the α-carboxyl group or
optionally through the ω-carboxyl group when the amino acid
is aspartic acid or glutamic acid or a peptide derived from
D, L or mixed D and L amino acids linked through a carboxyl
group, wherein the substituent groups are selected from
hydroxyl, amino, alkoxy, carboxy, benzyl, phenyl, nitro,
thiol, thioalkoxy, halogen, alkyl, aryl, alkenyl and
alkynyl;

T_5 is -OH, $-N(Z_1)Z_2$, R_5, D or L α-amino acid linked via
the α-amino group or optionally through the ω-amino group
when the amino acid is lysine or ornithine or a peptide
derived from D, L or mixed D and L amino acids linked
through an amino group, a chemical functional group, a
reporter group or a conjugate group;

Z_1 is hydrogen, C_1-C_6 alkyl, or an amino protecting
group;

Z_2 is hydrogen, C_1-C_6 alkyl, an amino protecting group,
$-C(=O)-(CH_2)_n-J-Z_3$, a D or L α-amino acid linked via the α-
carboxyl group or optionally through the ω-carboxyl group
when the amino acid is aspartic acid or glutamic acid or a
peptide derived from D, L or mixed D and L amino acids
linked through a carboxyl group;

Z_3 is hydrogen, an amino protecting group, $-C_1-C_6$ alkyl, $-C(=O)-CH_3$, benzyl, benzoyl, or $-(CH_2)_n-N(H)Z_1$;

each J is O, S or NH;

R_5 is a carbonyl protecting group; and

n is from 2 to about 50.

Another class of oligonucleotide mimetic that has been studied is based on linked morpholino units (morpholino nucleic acid) having heterocyclic bases attached to the morpholino ring. A number of linking groups have been reported that link the morpholino monomeric units in a morpholino nucleic acid. A preferred class of linking groups have been selected to give a non-ionic oligomeric compound. The non-ionic morpholino-based oligomeric compounds are less likely to have undesired interactions with cellular proteins. Morpholino-based oligomeric compounds are non-ionic mimics of oligonucleotides which are less likely to form undesired interactions with cellular proteins (Dwaine A. Braasch and David R. Corey, Biochemistry, 2002, 41(14), 4503-4510). Morpholino-based oligomeric compounds are disclosed in United States Patent 5,034,506, issued July 23, 1991. The morpholino class of oligomeric compounds have been prepared having a variety of different linking groups joining the monomeric subunits.

Morpholino nucleic acids have been prepared having a variety of different linking groups (L_2) joining the monomeric subunits. The basic formula is shown below:
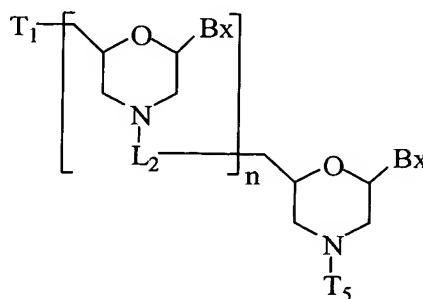
wherein

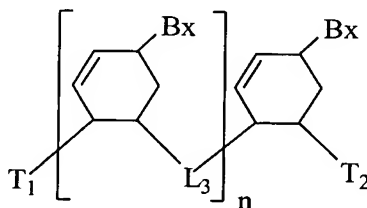T$_1$ is hydroxyl or a protected hydroxyl;

T$_5$ is hydrogen or a phosphate or phosphate derivative;

L$_2$ is a linking group; and

n is from 2 to about 50.

A further class of oligonucleotide mimetic is referred to as cyclohexenyl nucleic acids (CeNA). The furanose ring normally present in an DNA/RNA molecule is replaced with a cyclohenyl ring. CeNA DMT protected phosphoramidite monomers have been prepared and used for oligomeric compound synthesis following classical phosphoramidite chemistry. Fully modified CeNA oligomeric compounds and oligonucleotides having specific positions modified with CeNA have been prepared and studied (see Wang *et al.*, *J. Am. Chem. Soc.*, **2000**, *122*, 8595-8602). In general the incorporation of CeNA monomers into a DNA chain increases its stability of a DNA/RNA hybrid. CeNA oligoadenylates formed complexes with RNA and DNA complements with similar stability to the native complexes. The study of incorporating CeNA structures into natural nucleic acid structures was shown by NMR and circular dichroism to proceed with easy conformational adaptation. Furthermore the incorporation of CeNA into a sequence targeting RNA was stable to serum and able to activate E. Coli RNase resulting in cleavage of the target RNA strand.

The general formula of CeNA is shown below:

wherein

each Bx is a heterocyclic base moiety;

T$_1$ is hydroxyl or a protected hydroxyl; and

T2 is hydroxyl or a protected hydroxyl.

Another class of oligonucleotide mimetic (anhydrohexitol nucleic acid) can be prepared from one or more anhydrohexitol nucleosides (see, Wouters and Herdewijn, *Bioorg. Med. Chem. Lett.*, **1999**, **9**, 1563-1566) and would have the general formula:
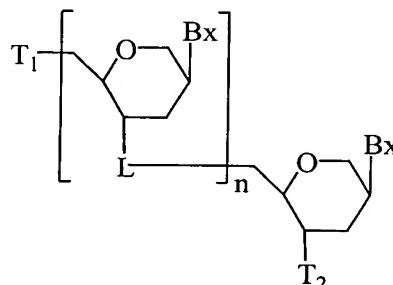
A further preferred modification includes Locked Nucleic Acids (LNAs) in which the 2'-hydroxyl group is linked to the 4' carbon atom of the sugar ring thereby forming a 2'-C,4'-C-oxymethylene linkage thereby forming a bicyclic sugar moiety. The linkage is preferably a methylene $(-CH_2-)_n$ group bridging the 2' oxygen atom and the 4' carbon atom wherein n is 1 or 2 (Singh et al., Chem. Commun., 1998, 4, 455-456). LNA and LNA analogs display very high duplex thermal stabilities with complementary DNA and RNA (Tm = +3 to +10 C), stability towards 3'-exonucleolytic degradation and good solubility properties. The basic structure of LNA showing the bicyclic ring system is shown below:
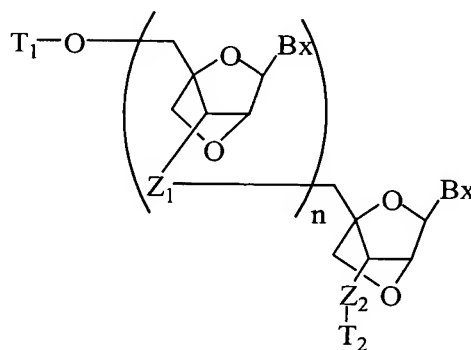
The conformations of LNAs determined by 2D NMR spectroscopy have shown that the locked orientation of the LNA nucleotides, both in single-stranded LNA and in duplexes, constrains the phosphate backbone in such a way

5   as to introduce a higher population of the N-type conformation (Petersen et al., J. Mol. Recognit., 2000, 13, 44-53). These conformations are associated with improved stacking of the nucleobases (Wengel et al., Nucleosides Nucleotides, 1999, 18, 1365-1370).

10   LNA has been shown to form exceedingly stable LNA:LNA duplexes (Koshkin et al., J. Am. Chem. Soc., 1998, 120, 13252-13253). LNA:LNA hybridization was shown to be the most thermally stable nucleic acid type duplex system, and the RNA-mimicking character of LNA was established at the

15   duplex level. Introduction of 3 LNA monomers (T or A) significantly increased melting points (Tm = +15/+11) toward DNA complements. The universality of LNA-mediated hybridization has been stressed by the formation of exceedingly stable LNA:LNA duplexes. The RNA-mimicking of

20   LNA was reflected with regard to the N-type conformational restriction of the monomers and to the secondary structure of the LNA:RNA duplex.

        LNAs also form duplexes with complementary DNA, RNA or LNA with high thermal affinities. Circular dichroism (CD)

25   spectra show that duplexes involving fully modified LNA (esp. LNA:RNA) structurally resemble an A-form RNA:RNA duplex. Nuclear magnetic resonance (NMR) examination of an LNA:DNA duplex confirmed the 3'-endo conformation of an LNA monomer. Recognition of double-stranded DNA has also been

30   demonstrated suggesting strand invasion by LNA. Studies of mismatched sequences show that LNAs obey the Watson-Crick base pairing rules with generally improved selectivity compared to the corresponding unmodified reference strands.

Novel types of LNA-oligomeric compounds, as well as the LNAs, are useful in a wide range of diagnostic and therapeutic applications. Among these are antisense applications, PCR applications, strand-displacement

5   oligomers, substrates for nucleic acid polymerases and generally as nucleotide based drugs. Potent and nontoxic antisense oligonucleotides containing LNAs have been described (Wahlestedt et al., Proc. Natl. Acad. Sci. U. S. A., 2000, 97, 5633-5638.) The authors have demonstrated

10   that LNAs confer several desired properties to antisense agents. LNA/DNA copolymers were not degraded readily in blood serum and cell extracts. LNA/DNA copolymers exhibited potent antisense activity in assay systems as disparate as G-protein-coupled receptor signaling in living

15   rat brain and detection of reporter genes in Escherichia coli. Lipofectin-mediated efficient delivery of LNA into living human breast cancer cells has also been accomplished.

The synthesis and preparation of the LNA monomers

20   adenine, cytosine, guanine, 5-methyl-cytosine, thymine and uracil, along with their oligomerization, and nucleic acid recognition properties have been described (Koshkin et al., Tetrahedron, 1998, 54, 3607-3630). LNAs and preparation thereof are also described in WO 98/39352 and WO 99/14226.

25   The first analogs of LNA, phosphorothioate-LNA and 2'-thio-LNAs, have also been prepared (Kumar et al., Bioorg. Med. Chem. Lett., 1998, 8, 2219-2222). Preparation of locked nucleoside analogs containing oligodeoxyribonucleotide duplexes as substrates for nucleic

30   acid polymerases has also been described (Wengel et al., PCT International Application WO 98-DK393 19980914). Furthermore, synthesis of 2'-amino-LNA, a novel conformationally restricted high-affinity oligonucleotide analog with a handle has been described in the art (Singh

et al., J. Org. Chem., 1998, 63, 10035-10039). In
addition, 2'-Amino- and 2'-methylamino-LNA's have been
prepared and the thermal stability of their duplexes with
complementary RNA and DNA strands has been previously
5   reported.

Further oligonucleotide mimetics have been prepared to
include bicyclic and tricyclic nucleoside analogs having
the formulas (amidite monomers shown):
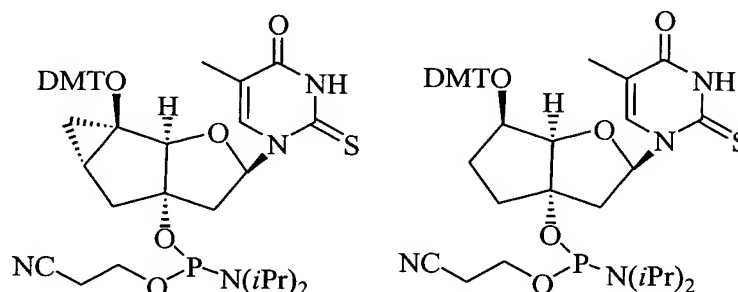
10

(see Steffens et al., Helv. Chim. Acta, 1997, 80, 2426-
2439; Steffens et al., J. Am. Chem. Soc., 1999, 121, 3249-
3255; and Renneberg et al., J. Am. Chem. Soc., 2002, 124,
5993-6002). These modified nucleoside analogs have been
15   oligomerized using the phosphoramidite approach and the
resulting oligomeric compounds containing tricyclic
nucleoside analogs have shown increased thermal stabilities
(Tm's) when hybridized to DNA, RNA and itself. Oligomeric
compounds containing bicyclic nucleoside analogs have shown
20   thermal stabilities approaching that of DNA duplexes.

Another class of oligonucleotide mimetic is referred
to as phosphonomonoester nucleic acids incorporate a
phosphorus group in a backbone the backbone. This class of
olignucleotide mimetic is reported to have useful physical
25   and biological and pharmacological properties in the areas
of inhibiting gene expression (antisense oligonucleotides,
ribozymes, sense oligonucleotides and triplex-forming
oligonucleotides), as probes for the detection of nucleic
acids and as auxiliaries for use in molecular biology.

The general formula (for definitions of Markush variables see: United States Patents 5,874,553 and 6,127,346 herein incorporated by reference in their entirety) is shown below.

$$Q-X-\underset{\underset{R_6}{\overset{Z}{\underset{|}{P}}}}{\overset{Z}{\underset{|}{\overset{||}{P}}}}-\underset{R_6}{\overset{R_5 \overset{A-B}{\underset{|}{\overset{|}{L}}}}{\underset{|}{C}}}-D-G-X-\left[\underset{\underset{R_6}{\overset{Z}{\underset{|}{P}}}}{\overset{Z}{\underset{|}{\overset{||}{P}}}}-\underset{R_6}{\overset{R_5 \overset{A-B}{\underset{|}{\overset{|}{L}}}}{\underset{|}{C}}}-D-G\right]_n X-Q'$$
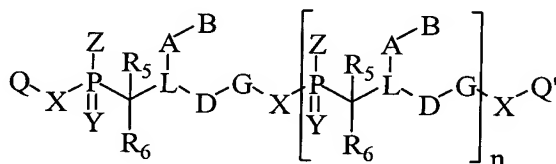
Another oligonucleotide mimetic has been reported wherein the furanosyl ring has been replaced by a cyclobutyl moiety.

Modified sugars

Oligomeric compounds of the invention may also contain one or more substituted sugar moieties. Preferred oligomeric compounds comprise a sugar substituent group selected from: OH; F; O-, S-, or N-alkyl; O-, S-, or N-alkenyl; O-, S- or N-alkynyl; or O-alkyl-O-alkyl, wherein the alkyl, alkenyl and alkynyl may be substituted or unsubstituted C_1 to C_{10} alkyl or C_2 to C_{10} alkenyl and alkynyl. Particularly preferred are $O[(CH_2)_nO]_mCH_3$, $O(CH_2)_nOCH_3$, $O(CH_2)_nNH_2$, $O(CH_2)_nCH_3$, $O(CH_2)_nONH_2$, and $O(CH_2)_nON[(CH_2)_nCH_3]_2$, where n and m are from 1 to about 10. Other preferred oligonucleotides comprise a sugar substituent group selected from: C_1 to C_{10} lower alkyl, substituted lower alkyl, alkenyl, alkynyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH_3, OCN, Cl, Br, CN, CF_3, OCF_3, $SOCH_3$, SO_2CH_3, ONO_2, NO_2, N_3, NH_2, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of an oligonucleotide, or a group for improving the pharmacodynamic properties of an oligonucleotide, and other substituents having similar properties. A preferred modification includes 2'-methoxyethoxy (2'-O-$CH_2CH_2OCH_3$, also

known as 2'-O-(2-methoxyethyl) or 2'-MOE) (Martin *et al.,*
Helv. Chim. Acta, **1995,** *78,* 486-504) i.e., an alkoxyalkoxy
group. A further preferred modification includes 2'-
dimethylaminooxyethoxy, i.e., a $O(CH_2)_2ON(CH_3)_2$ group, also
5 known as 2'-DMAOE, as described in examples hereinbelow,
and 2'-dimethylaminoethoxyethoxy (also known in the art as
2'-O-dimethyl-amino-ethoxy-ethyl or 2'-DMAEOE), i.e., 2'-O-
CH_2-O-CH_2-N(CH_3)$_2$.

Other preferred sugar substituent groups include
10 methoxy (-O-CH_3), aminopropoxy (-$OCH_2CH_2CH_2NH_2$), allyl (-CH_2-
CH=CH_2), -O-allyl (-O-CH_2-CH=CH_2) and fluoro (F). 2'-Sugar
substituent groups may be in the arabino (up) position or
ribo (down) position. A preferred 2'-arabino modification
is 2'-F. Similar modifications may also be made at other
15 positions on the oligomeric compound, particularly the 3'
position of the sugar on the 3' terminal nucleoside or in
2'-5' linked oligonucleotides and the 5' position of 5'
terminal nucleotide. Oligomeric compounds may also have
sugar mimetics such as cyclobutyl moieties in place of the
20 pentofuranosyl sugar. Representative United States patents
that teach the preparation of such modified sugar
structures include, but are not limited to, U.S.:
4,981,957; 5,118,800; 5,319,080; 5,359,044; 5,393,878;
5,446,137; 5,466,786; 5,514,785; 5,519,134; 5,567,811;
25 5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,053;
5,639,873; 5,646,265; 5,658,873; 5,670,633; 5,792,747; and
5,700,920, certain of which are commonly owned with the
instant application, and each of which is herein
incorporated by reference in its entirety.

30   Further representative sugar substituent groups
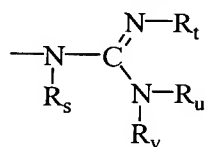include groups of formula I_a or II_a:

Ia   IIa
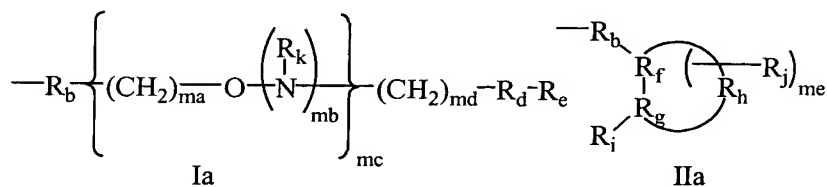
wherein:

R_b is O, S or NH;

R_d is a single bond, O, S or C(=O);

R_e is C_1-C_{10} alkyl, N(R_k)(R_m), N(R_k)(R_n), N=C(R_p)(R_q),

N=C(R_p)(R_r) or has formula III_a;

$$-N-C\begin{matrix} N-R_t \\ \\ N-R_u \\ | \\ R_v \end{matrix}$$
$$\underset{R_s}{|}$$

IIIa

R_p and R_q are each independently hydrogen or C_1-C_{10} alkyl;

R_r is -R_x-R_y;

each R_s, R_t, R_u and R_v is, independently, hydrogen, C(O)R_w, substituted or unsubstituted C_1-C_{10} alkyl, substituted or unsubstituted C_2-C_{10} alkenyl, substituted or unsubstituted C_2-C_{10} alkynyl, alkylsulfonyl, arylsulfonyl, a chemical functional group or a conjugate group, wherein the substituent groups are selected from hydroxyl, amino, alkoxy, carboxy, benzyl, phenyl, nitro, thiol, thioalkoxy, halogen, alkyl, aryl, alkenyl and alkynyl;

or optionally, R_u and R_v, together form a phthalimido moiety with the nitrogen atom to which they are attached;

each R_w is, independently, substituted or unsubstituted C_1-C_{10} alkyl, trifluoromethyl, cyanoethyloxy, methoxy, ethoxy, t-butoxy, allyloxy, 9-fluorenylmethoxy, 2-(trimethylsilyl)-ethoxy, 2,2,2-trichloroethoxy, benzyloxy, butyryl, iso-butyryl, phenyl or aryl;

R_k is hydrogen, a nitrogen protecting group or -R_x-R_y;

R_p is hydrogen, a nitrogen protecting group or -R_x-R_y;

R_x is a bond or a linking moiety;

R_y is a chemical functional group, a conjugate group or a solid support medium;

each R_m and R_n is, independently, H, a nitrogen protecting group, substituted or unsubstituted C_1-C_{10} alkyl, substituted or unsubstituted C_2-C_{10} alkenyl, substituted or unsubstituted C_2-C_{10} alkynyl, wherein the substituent groups are selected from hydroxyl, amino, alkoxy, carboxy, benzyl, phenyl, nitro, thiol, thioalkoxy, halogen, alkyl, aryl, alkenyl, alkynyl; NH_3^+, $N(R_u)(R_v)$, guanidino and acyl where said acyl is an acid amide or an ester;

or R_m and R_n, together, are a nitrogen protecting group, are joined in a ring structure that optionally includes an additional heteroatom selected from N and O or are a chemical functional group;

R_i is OR_z, SR_z, or $N(R_z)_2$;

each R_z is, independently, H, C_1-C_8 alkyl, C_1-C_8 haloalkyl, $C(=NH)N(H)R_u$, $C(=O)N(H)R_u$ or $OC(=O)N(H)R_u$;

R_f, R_g and R_h comprise a ring system having from about 4 to about 7 carbon atoms or having from about 3 to about 6 carbon atoms and 1 or 2 heteroatoms wherein said heteroatoms are selected from oxygen, nitrogen and sulfur and wherein said ring system is aliphatic, unsaturated aliphatic, aromatic, or saturated or unsaturated heterocyclic;

R_j is alkyl or haloalkyl having 1 to about 10 carbon atoms, alkenyl having 2 to about 10 carbon atoms, alkynyl having 2 to about 10 carbon atoms, aryl having 6 to about 14 carbon atoms, $N(R_k)(R_m)$ OR_k, halo, SR_k or CN;

m_a is 1 to about 10;

each mb is, independently, 0 or 1;

mc is 0 or an integer from 1 to 10;

md is an integer from 1 to 10;

me is from 0, 1 or 2; and

provided that when mc is 0, md is greater than 1.

Representative substituents groups of Formula I are disclosed in United States Patent Application Serial No. 09/130,973, filed August 7, 1998, entitled "Capped 2'-Oxyethoxy Oligonucleotides," hereby incorporated by reference in its entirety.

Representative cyclic substituent groups of Formula II are disclosed in United States Patent Application Serial No. 09/123,108, filed July 27, 1998, entitled "RNA Targeted 2'-Oligomeric compounds that are Conformationally Preorganized," hereby incorporated by reference in its entirety.

Particularly preferred sugar substituent groups include $O[(CH_2)_nO]_mCH_3$, $O(CH_2)_nOCH_3$, $O(CH_2)_nNH_2$, $O(CH_2)_nCH_3$, $O(CH_2)_nONH_2$, and $O(CH_2)_nON[(CH_2)_nCH_3)]_2$, where n and m are from 1 to about 10.

Representative guanidino substituent groups that are shown in formula III and IV are disclosed in co-owned United States Patent Application 09/349,040, entitled "Functionalized Oligomers", filed July 7, 1999, hereby incorporated by reference in its entirety.

Representative acetamido substituent groups are disclosed in United States Patent 6,147,200 which is hereby incorporated by reference in its entirety.

Representative dimethylaminoethyloxyethyl substituent groups are disclosed in International Patent Application PCT/US99/17895, entitled "2'-O-Dimethylaminoethyloxyethyl-Oligomeric compounds", filed August 6, 1999, hereby incorporated by reference in its entirety.

Modified Nucleobases/Naturally Occurring Nucleobases

Oligomeric compounds may also include nucleobase (often referred to in the art simply as "base" or "heterocyclic base moiety") modifications or substitutions.

As used herein, "unmodified" or "natural" nucleobases
include the purine bases adenine (A) and guanine (G), and
the pyrimidine bases thymine (T), cytosine (C) and uracil
(U). Modified nucleobases also referred herein as
5   heterocyclic base moieties include other synthetic and
natural nucleobases such as 5-methylcytosine (5-me-C), 5-
hydroxymethyl cytosine, xanthine, hypoxanthine, 2-
aminoadenine, 6-methyl and other alkyl derivatives of
adenine and guanine, 2-propyl and other alkyl derivatives
10   of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-
thiocytosine, 5-halouracil and cytosine, 5-propynyl (-C≡C-
CH$_3$) uracil and cytosine and other alkynyl derivatives of
pyrimidine bases, 6-azo uracil, cytosine and thymine, 5-
uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-
15   thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted
adenines and guanines, 5-halo particularly 5-bromo, 5-
trifluoromethyl and other 5-substituted uracils and cyto-
sines, 7-methylguanine and 7-methyladenine, 2-F-adenine, 2-
amino-adenine, 8-azaguanine and 8-azaadenine, 7-
20   deazaguanine and 7-deazaadenine and 3-deazaguanine and 3-
deazaadenine.

Heterocyclic base moieties may also include those in
which the purine or pyrimidine base is replaced with other
heterocycles, for example 7-deaza-adenine, 7-
25   deazaguanosine, 2-aminopyridine and 2-pyridone. Further
nucleobases include those disclosed in United States Patent
No. 3,687,808, those disclosed in *The Concise Encyclopedia
Of Polymer Science And Engineering*, pages 858-859,
Kroschwitz, J.I., ed. John Wiley & Sons, **1990**, those
30   disclosed by Englisch et al., *Angewandte Chemie*,
International Edition, **1991**, *30*, 613, and those disclosed
by Sanghvi, Y.S., Chapter 15, *Antisense Research and
Applications*, pages 289-302, Crooke, S.T. and Lebleu, B. ,
ed., CRC Press, **1993**. Certain of these nucleobases are
35   particularly useful for increasing the binding affinity of

the oligomeric compounds of the invention. These include
5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6
and O-6 substituted purines, including 2-aminopropyl-
adenine, 5-propynyluracil and 5-propynylcytosine. 5-
5   methylcytosine substitutions have been shown to increase
nucleic acid duplex stability by 0.6-1.2°C (Sanghvi, Y.S.,
Crooke, S.T. and Lebleu, B., eds., *Antisense Research and
Applications*, CRC Press, Boca Raton, **1993**, pp. 276-278) and
are presently preferred base substitutions, even more
10 particularly when combined with 2'-O-methoxyethyl sugar
modifications.

       In one aspect of the present invention oligomeric
compounds are prepared having polycyclic heterocyclic
compounds in place of one or more heterocyclic base
15 moieties. A number of tricyclic heterocyclic compounds
have been previously reported. These compounds are
routinely used in antisense applications to increase the
binding properties of the modified strand to a target
strand. The most studied modifications are targeted to
20 guanosines hence they have been termed G-clamps or cytidine
analogs. Many of these polycyclic heterocyclic compounds
have the general formula:

$$
\begin{array}{c}
R_{12} \\
R_{11} \quad \quad R_{13} \\
R_{14} \\
NH \quad R_{10} \\
N \\
R_{15} \quad N
\end{array}
$$
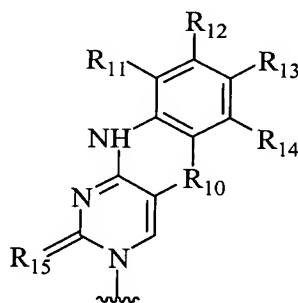
25   Representative cytosine analogs that make 3 hydrogen
bonds with a guanosine in a second strand include 1,3-
diazaphenoxazine-2-one ($R_{10} = O$, R_{11} - $R_{14}= H$) [Kurchavov, *et
al.*, *Nucleosides and Nucleotides*, 1997, 16, 1837-1846],
1,3-diazaphenothiazine-2-one ($R_{10}= S$, R_{11} - $R_{14}= H$), [Lin,

K.-Y.; Jones, R. J.; Matteucci, M. J. Am. Chem. Soc. 1995, 117, 3873-3874] and 6,7,8,9-tetrafluoro-1,3-diazaphenoxazine-2-one (R_{10} = O, R_{11} - R_{14} = F) [Wang, J.; Lin, K.-Y., Matteucci, M. Tetrahedron Lett. 1998, 39, 8385-

5   8388]. Incorporated into oligonucleotides these base modifications were shown to hybridize with complementary guanine and the latter was also shown to hybridize with adenine and to enhance helical thermal stability by extended stacking interactions(also see U.S. Patent

10   Application entitled "Modified Peptide Nucleic Acids" filed May 24, 2002, Serial number 10/155,920; and U.S. Patent Application entitled "Nuclease Resistant Chimeric Oligonucleotides" filed May 24, 2002, Serial number 10/013,295, both of which are commonly owned with this

15   application and are herein incorporated by reference in their entirety).

Further helix-stabilizing properties have been observed when a cytosine analog/substitute has an aminoethoxy moiety attached to the rigid 1,3-diazaphenox-

20   azine-2-one scaffold (R_{10} = O, R_{11} = -O-$(CH_2)_2$-NH_2, R_{12-14}=H) [Lin, K.-Y.; Matteucci, M. J. Am. Chem. Soc. 1998, 120, 8531-8532]. Binding studies demonstrated that a single incorporation could enhance the binding affinity of a model oligonucleotide to its complementary target DNA or RNA with

25   a ΔT_m of up to 18° relative to 5-methyl cytosine (dC5^{me}), which is the highest known affinity enhancement for a single modification, yet. On the other hand, the gain in helical stability does not compromise the specificity of the oligonucleotides. The T_m data indicate an even greater

30   discrimination between the perfect match and mismatched sequences compared to dC5^{me}. It was suggested that the tethered amino group serves as an additional hydrogen bond donor to interact with the Hoogsteen face, namely the O6, of a complementary guanine thereby forming 4 hydrogen

35   bonds. This means that the increased affinity of G-clamp is mediated by the combination of extended base stacking and additional specific hydrogen bonding.

Further tricyclic heterocyclic compounds and methods of using them that are amenable to the present invention are disclosed in United States Patent Serial Number 6,028,183, which issued on May 22, 2000, and United States

5   Patent Serial Number 6,007,992, which issued on December 28, 1999, the contents of both are commonly assigned with this application and are incorporated herein in their entirety.

The enhanced binding affinity of the phenoxazine

10 derivatives together with their uncompromised sequence specificity makes them valuable nucleobase analogs for the development of more potent antisense-based drugs. In fact, promising data have been derived from in vitro experiments demonstrating that heptanucleotides containing phenoxazine

15 substitutions are capable to activate RNaseH, enhance cellular uptake and exhibit an increased antisense activity [Lin, K-Y; Matteucci, M. J. Am. Chem. Soc. 1998, 120, 8531-8532]. The activity enhancement was even more pronounced in case of G-clamp, as a single substitution was shown to

20 significantly improve the in vitro potency of a 20mer 2'-deoxyphosphorothioate oligonucleotides [Flanagan, W. M.; Wolf, J.J.; Olson, P.; Grant, D.; Lin, K.-Y.; Wagner, R. W.; Matteucci, M. Proc. Natl. Acad. Sci. USA, 1999, 96, 3513-3518]. Nevertheless, to optimize oligonucleotide

25 design and to better understand the impact of these heterocyclic modifications on the biological activity, it is important to evaluate their effect on the nuclease stability of the oligomers.

Further modified polycyclic heterocyclic compounds

30 useful as heterocyclcic bases are disclosed in but not limited to, the above noted U.S. 3,687,808, as well as U.S.: 4,845,205; 5,130,302; 5,134,066; 5,175,273; 5,367,066; 5,432,272; 5,434,257; 5,457,187; 5,459,255; 5,484,908; 5,502,177; 5,525,711; 5,552,540; 5,587,469;

35 5,594,121, 5,596,091; 5,614,617; 5,645,985; 5,646,269; 5,750,692; 5,830,653; 5,763,588; 6,005,096; and 5,681,941, and Unites States Patent Application Serial number

09/996,292 filed November 28, 2001, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference.

5   The oligonucleotides of the present invention also include variants in which a different base is present at one or more of the nucleotide positions in the oligonucleotide. For example, if the first nucleotide is an adenosine, variants may be produced which contain thymidine, guanosine or cytidine at this position. This

10   may be done at any of the positions of the oligonucleotide. Thus, a 20-mer may comprise 60 variations (20 positions x 3 alternates at each position) in which the original nucleotide is substituted with any of the three alternate nucleotides. These oligonucleotides are then tested using

15   the methods described herein to determine their ability to inhibit expression of HCV mRNA and/or HCV replication.

Conjugates

A further preferred substitution that can be appended

20   to the oligomeric compounds of the invention involves the linkage of one or more moieties or conjugates which enhance the activity, cellular distribution or cellular uptake of the resulting oligomeric compounds. In one embodiment such modified oligomeric compounds are prepared by covalently

25   attaching conjugate groups to functional groups such as hydroxyl or amino groups. Conjugate groups of the invention include intercalators, reporter molecules, polyamines, polyamides, polyethylene glycols, polyethers, groups that enhance the pharmacodynamic properties of

30   oligomers, and groups that enhance the pharmacokinetic properties of oligomers. Typical conjugates groups include cholesterols, lipids, phospholipids, biotin, phenazine, folate, phenanthridine, anthraquinone, acridine, fluores-ceins, rhodamines, coumarins, and dyes. Groups that

35   enhance the pharmacodynamic properties, in the context of this invention, include groups that improve oligomer uptake, enhance oligomer resistance to degradation, and/or

strengthen sequence-specific hybridization with RNA.
Groups that enhance the pharmacokinetic properties, in the
context of this invention, include groups that improve
oligomer uptake, distribution, metabolism or excretion.

5   Representative conjugate groups are disclosed in
International Patent Application PCT/US92/09196, filed
October 23, 1992 the entire disclosure of which is incor-
porated herein by reference. Conjugate moieties include
but are not limited to lipid moieties such as a cholesterol

10 moiety (Letsinger et al., *Proc. Natl. Acad. Sci. USA*, **1989**,
86, 6553-6556), cholic acid (Manoharan et al., *Bioorg. Med.
Chem. Let.*, **1994**, *4*, 1053-1060), a thioether, e.g., hexyl-
S-tritylthiol (Manoharan et al., *Ann. N.Y. Acad. Sci.*,
1992, *660*, 306-309; Manoharan et al., *Bioorg. Med. Chem.*

15 *Let.*, **1993**, *3*, 2765-2770), a thiocholesterol (Oberhauser et
al., *Nucl. Acids Res.*, **1992**, *20*, 533-538), an aliphatic
chain, e.g., dodecandiol or undecyl residues (Saison-
Behmoaras et al., *EMBO J.*, **1991**, *10*, 1111-1118; Kabanov et
al., *FEBS Lett.*, **1990**, *259*, 327-330; Svinarchuk et al.,

20 *Biochimie*, **1993**, *75*, 49-54), a phospholipid, e.g., di-
hexadecyl-rac-glycerol or triethylammonium 1,2-di-O-
hexadecyl-rac-glycero-3-H-phosphonate (Manoharan et al.,
Tetrahedron Lett., **1995**, *36*, 3651-3654; Shea et al., *Nucl.
Acids Res.*, **1990**, *18*, 3777-3783), a polyamine or a

25 polyethylene glycol chain (Manoharan et al., *Nucleosides &
Nucleotides*, **1995**, *14*, 969-973), or adamantane acetic acid
(Manoharan et al., *Tetrahedron Lett.*, **1995**, *36*, 3651-3654),
a palmityl moiety (Mishra et al., *Biochim. Biophys. Acta*,
1995, *1264*, 229-237), or an octadecylamine or hexylamino-

30 carbonyl-oxycholesterol moiety (Crooke et al., *J.
Pharmacol. Exp. Ther.*, **1996**, *277*, 923-937.

     The oligomeric compounds of the invention may also be
conjugated to active drug substances, for example, aspirin,

warfarin, phenylbutazone, ibuprofen, suprofen, fenbufen, ketoprofen, (S)-(+)-pranoprofen, carprofen, dansylsarcosine, 2,3,5-triiodobenzoic acid, flufenamic acid, folinic acid, a benzothiadiazide, chlorothiazide, a

5 diazepine, indomethicin, a barbiturate, a cephalosporin, a sulfa drug, an antidiabetic, an antibacterial or an antibiotic. Oligonucleotide-drug conjugates and their preparation are described in United States Patent Application 09/334,130 (filed June 15, 1999) which is

10 incorporated herein by reference in its entirety.

Representative United States patents that teach the preparation of such oligonucleotide conjugates include, but are not limited to, U.S.: 4,828,979; 4,948,882; 5,218,105; 5,525,465; 5,541,313; 5,545,730; 5,552,538; 5,578,717,

15 5,580,731; 5,580,731; 5,591,584; 5,109,124; 5,118,802; 5,138,045; 5,414,077; 5,486,603; 5,512,439; 5,578,718; 5,608,046; 4,587,044; 4,605,735; 4,667,025; 4,762,779; 4,789,737; 4,824,941; 4,835,263; 4,876,335; 4,904,582; 4,958,013; 5,082,830; 5,112,963; 5,214,136; 5,082,830;

20 5,112,963; 5,214,136; 5,245,022; 5,254,469; 5,258,506; 5,262,536; 5,272,250; 5,292,873; 5,317,098; 5,371,241, 5,391,723; 5,416,203, 5,451,463; 5,510,475; 5,512,667; 5,514,785; 5,565,552; 5,567,810; 5,574,142; 5,585,481; 5,587,371; 5,595,726; 5,597,696; 5,599,923; 5,599,928 and

25 5,688,941, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference.

Chimeric Oligomeric Compounds

30 It is not necessary for all positions in an oligomeric compound to be uniformly modified, and in fact more than one of the aforementioned modifications may be incorporated in a single oligomeric compound or even at a single monomeric subunit such as a nucleoside within a oligomeric

35 compound. The present invention also includes oligomeric compounds which are chimeric oligomeric compounds. "Chimeric" oligomeric compounds or "chimeras," in the

context of this invention, are oligomeric compounds that contain two or more chemically distinct regions, each made up of at least one monomer unit, i.e., a nucleotide in the case of a nucleic acid based oligomer.

Chimeric oligomeric compounds typically contain at least one region modified so as to confer increased resistance to nuclease degradation, increased cellular uptake, and/or increased binding affinity for the target nucleic acid. An additional region of the oligomeric compound may serve as a substrate for enzymes capable of cleaving RNA:DNA or RNA:RNA hybrids. By way of example, RNase H is a cellular endonuclease which cleaves the RNA strand of an RNA:DNA duplex. Activation of RNase H, therefore, results in cleavage of the RNA target, thereby greatly enhancing the efficiency of inhibition of gene expression. Consequently, comparable results can often be obtained with shorter oligomeric compounds when chimeras are used, compared to for example phosphorothioate deoxyoligonucleotides hybridizing to the same target region. Cleavage of the RNA target can be routinely detected by gel electrophoresis and, if necessary, associated nucleic acid hybridization techniques known in the art.

Chimeric oligomeric compounds of the invention may be formed as composite structures of two or more oligonucleotides, oligonucleotide analogs, oligonucleosides and/or oligonucleotide mimetics as described above. Such oligomeric compounds have also been referred to in the art as hybrids hemimers, gapmers or inverted gapmers. Representative United States patents that teach the preparation of such hybrid structures include, but are not limited to, U.S.: 5,013,830; 5,149,797; 5,220,007; 5,256,775; 5,366,878; 5,403,711; 5,491,133; 5,565,350; 5,623,065; 5,652,355; 5,652,356; and 5,700,922, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference in its entirety.

3'-endo modifications

In one aspect of the present invention oligomeric compounds include nucleosides synthetically modified to induce a 3'-endo sugar conformation. A nucleoside can
5   incorporate synthetic modifications of the heterocyclic base, the sugar moiety or both to induce a desired 3'-endo sugar conformation. These modified nucleosides are used to mimic RNA like nucleosides so that particular properties of an oligomeric compound can be enhanced while maintaining
10 the desirable 3'-endo conformational geometry. There is an apparent preference for an RNA type duplex (A form helix, predominantly 3'-endo) as a requirement (e.g. trigger) of RNA interference which is supported in part by the fact that duplexes composed of 2'-deoxy-2'-F-nucleosides appears
15 efficient in triggering RNAi response in the *C. elegans* system. Properties that are enhanced by using more stable 3'-endo nucleosides include but aren't limited to modulation of pharmacokinetic properties through modification of protein binding, protein off-rate,
20 absorption and clearance; modulation of nuclease stability as well as chemical stability; modulation of the binding affinity and specificity of the oligomer (affinity and specificity for enzymes as well as for complementary sequences); and increasing efficacy of RNA cleavage. The
25 present invention provides oligomeric triggers of RNAi having one or more nucleosides modified in such a way as to favor a C3'-endo type conformation.

Scheme 1

30   C2'-endo/Southern   C3'-endo/Northern
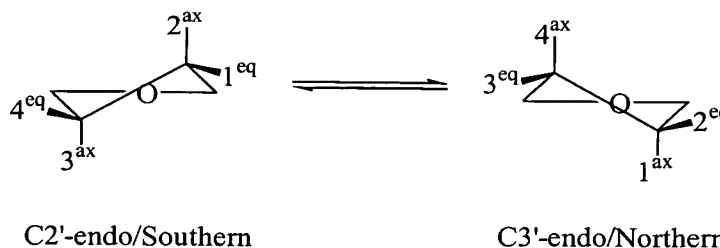
Nucleoside conformation is influenced by various factors including substitution at the 2', 3' or 4'-positions of the pentofuranosyl sugar. Electronegative substituents generally prefer the axial positions, while

5 sterically demanding substituents generally prefer the equatorial positions (Principles of Nucleic Acid Structure, Wolfgang Sanger, 1984, Springer-Verlag.) Modification of the 2' position to favor the 3'-endo conformation can be achieved while maintaining the 2'-OH as a recognition

10 element, as illustrated in Figure 2, below (Gallo et al., Tetrahedron (2001), 57, 5707-5713. Harry-O'kuru et al., J. Org. Chem., (1997), 62(6), 1754-1759 and Tang et al., J. Org. Chem. (1999), 64, 747-754.) Alternatively, preference for the 3'-endo conformation can be achieved by deletion of

15 the 2'-OH as exemplified by 2'deoxy-2'F-nucleosides (Kawasaki et al., J. Med. Chem. (1993), 36, 831-841), which adopts the 3'-endo conformation positioning the electronegative fluorine atom in the axial position. Other modifications of the ribose ring, for example substitution

20 at the 4'-position to give 4'-F modified nucleosides (Guillerm et al., Bioorganic and Medicinal Chemistry Letters (1995), 5, 1455-1460 and Owen et al., J. Org. Chem. (1976), 41, 3010-3017), or for example modification to yield methanocarba nucleoside analogs (Jacobson et al., J.

25 Med. Chem. Lett. (2000), 43, 2196-2203 and Lee et al., Bioorganic and Medicinal Chemistry Letters (2001), 11, 1333-1337) also induce preference for the 3'-endo conformation. Along similar lines, oligomeric triggers of RNAi response might be composed of one or more nucleosides

30 modified in such a way that conformation is locked into a C3'-endo type conformation, i.e. Locked Nucleic Acid (LNA, Singh et al, Chem. Commun. (1998), 4, 455-456), and ethylene bridged Nucleic Acids (ENA, Morita et al, Bioorganic & Medicinal Chemistry Letters (2002), 12, 73-

35 76.) Examples of modified nucleosides amenable to the present invention are shown below in Table I. These examples are meant to be representative and not exhaustive.

Table I
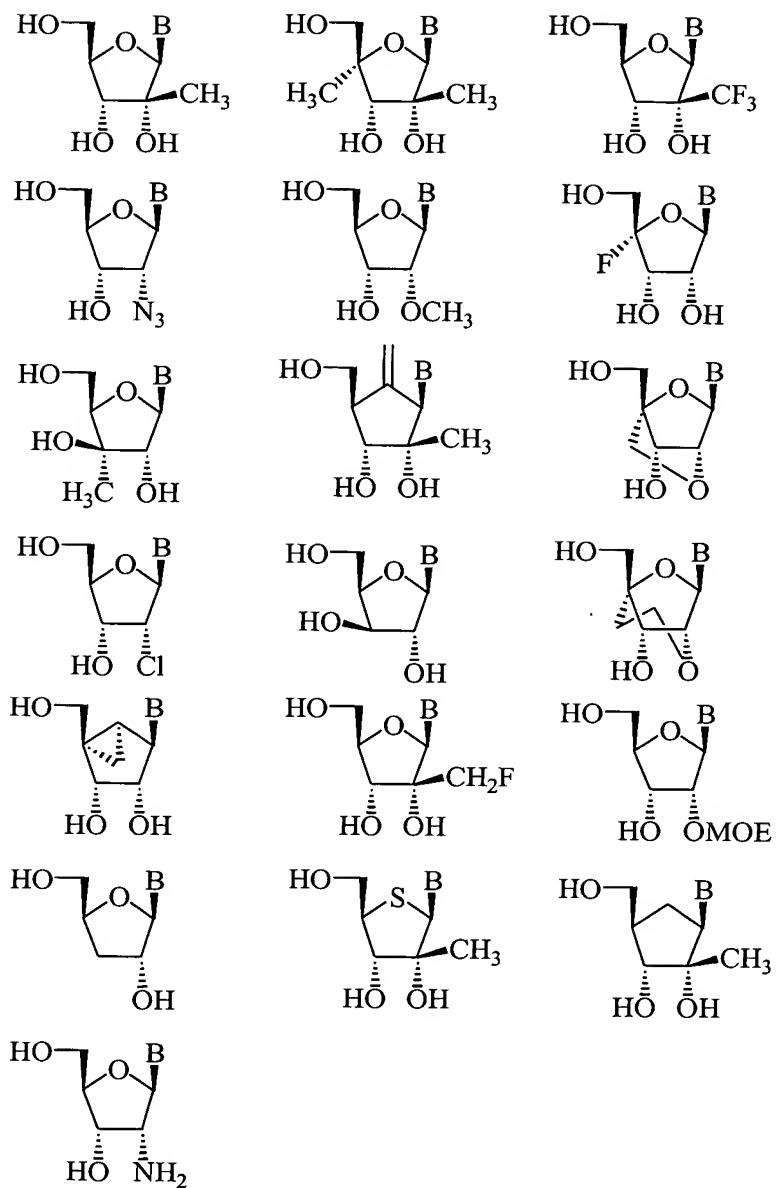
5   The preferred conformation of modified nucleosides and their oligomers can be estimated by various methods such as molecular dynamics calculations, nuclear magnetic resonance spectroscopy and CD measurements. Hence, modifications predicted to induce RNA like conformations, A-form duplex

geometry in an oligomeric context, are selected for use in
the modified oligoncleotides of the present invention. The
synthesis of numerous of the modified nucleosides amenable
to the present invention are known in the art (see for
5 example, Chemistry of Nucleosides and Nucleotides Vol 1-3,
ed. Leroy B. Townsend, 1988, Plenum press., and the
examples section below.) Nucleosides known to be
inhibitors/substrates for RNA dependent RNA polymerases
(for example HCV NS5B

10   In one aspect, the present invention is directed to
oligonucleotides that are prepared having enhanced
properties compared to native RNA against nucleic acid
targets. A target is identified and an oligonucleotide is
selected having an effective length and sequence that is
15 complementary to a portion of the target sequence. Each
nucleoside of the selected sequence is scrutinized for
possible enhancing modifications. A preferred modification
would be the replacement of one or more RNA nucleosides
with nucleosides that have the same 3'-endo conformational
20 geometry. Such modifications can enhance chemical and
nuclease stability relative to native RNA while at the same
time being much cheaper and easier to synthesize and/or
incorporate into an oligonulceotide. The selected sequence
can be further divided into regions and the nucleosides of
25 each region evaluated for enhancing modifications that can
be the result of a chimeric configuration. Consideration
is also given to the 5' and 3'-termini as there are often
advantageous modifications that can be made to one or more
of the terminal nucleosides. The oligomeric compounds of
30 the present invention include at least one 5'-modified
phosphate group on a single strand or on at least one 5'-
position of a double stranded sequence or sequences.
Further modifications are also considered such as
internucleoside linkages, conjugate groups, substitute
35 sugars or bases, substitution of one or more nucleosides
with nucleoside mimetics and any other modification that
can enhance the selected sequence for its intended target.

The terms used to describe the conformational geometry of homoduplex nucleic acids are "A Form" for RNA and ''B Form'' for DNA. The respective conformational geometry for RNA and DNA duplexes was determined from X-ray diffraction

5   analysis of nucleic acid fibers (Arnott and Hukins, *Biochem. Biophys. Res. Comm.*, **1970**, 47, 1504.) In general, RNA:RNA duplexes are more stable and have higher melting temperatures (Tm's) than DNA:DNA duplexes (Sanger et al., Principles of Nucleic Acid Structure, 1984, Springer-

10   Verlag; New York, NY.; Lesnik et al., Biochemistry, 1995, 34, 10807-10815; Conte et al., Nucleic Acids Res., 1997, 25, 2627-2634). The increased stability of RNA has been attributed to several structural features, most notably the improved base stacking interactions that result from an A-

15   form geometry (Searle et al., Nucleic Acids Res., 1993, 21, 2051-2056). The presence of the 2' hydroxyl in RNA biases the sugar toward a C3' endo pucker, i.e., also designated as Northern pucker, which causes the duplex to favor the A-form geometry. In addition, the 2' hydroxyl groups of RNA

20   can form a network of water mediated hydrogen bonds that help stabilize the RNA duplex (Egli et al., Biochemistry, 1996, 35, 8489-8494). On the other hand, deoxy nucleic acids prefer a C2' endo sugar pucker, i.e., also known as Southern pucker, which is thought to impart a less stable

25   B-form geometry (Sanger, W. (1984) Principles of Nucleic Acid Structure, Springer-Verlag, New York, NY). As used herein, B-form geometry is inclusive of both C2'-endo pucker and O4'-endo pucker. This is consistent with Berger, *et. al.*, *Nucleic Acids Research*, **1998**, *26*, 2473-

30   2480, who pointed out that in considering the furanose conformations which give rise to B-form duplexes consideration should also be given to a O4'-endo pucker contribution.

    DNA:RNA hybrid duplexes, however, are usually less

35   stable than pure RNA:RNA duplexes, and depending on their sequence may be either more or less stable than DNA:DNA

duplexes (Searle *et al.*, *Nucleic Acids Res.*, **1993**, *21*, 2051-2056). The structure of a hybrid duplex is intermediate between A- and B-form geometries, which may result in poor stacking interactions (Lane *et al.*, *Eur. J.*

5   *Biochem.*, **1993**, *215*, 297-306; Fedoroff *et al.*, *J. Mol. Biol.*, **1993**, *233*, 509-523; Gonzalez *et al.*, *Biochemistry*, **1995**, *34*, 4969-4982; Horton *et al.*, *J. Mol. Biol.*, **1996**, *264*, 521-533). The stability of the duplex formed between a target RNA and a synthetic sequence is central to

10 therapies such as but not limited to antisense and RNA interference as these mechanisms require the binding of a synthetic oligonucleotide strand to an RNA target strand. In the case of antisense, effective inhibition of the mRNA requires that the antisense DNA have a very high binding

15 affinity with the mRNA. Otherwise the desired interaction between the synthetic oligonucleotide strand and target mRNA strand will occur infrequently, resulting in decreased efficacy.

One routinely used method of modifying the sugar

20 puckering is the substitution of the sugar at the 2'-position with a substituent group that influences the sugar geometry. The influence on ring conformation is dependant on the nature of the substituent at the 2'-position. A number of different substituents have been studied to

25 determine their sugar puckering effect. For example, 2'-halogens have been studied showing that the 2'-fluoro derivative exhibits the largest population (65%) of the C3'-endo form, and the 2'-iodo exhibits the lowest population (7%). The populations of adenosine (2'-OH)

30 versus deoxyadenosine (2'-H) are 36% and 19%, respectively. Furthermore, the effect of the 2'-fluoro group of adenosine dimers (2'-deoxy-2'-fluoroadenosine - 2'-deoxy-2'-fluoro-adenosine) is further correlated to the stabilization of the stacked conformation.

35 As expected, the relative duplex stability can be enhanced by replacement of 2'-OH groups with 2'-F groups

thereby increasing the C3'-endo population. It is assumed that the highly polar nature of the 2'-F bond and the extreme preference for C3'-endo puckering may stabilize the stacked conformation in an A-form duplex. Data from UV
5   hypochromicity, circular dichroism, and ^{1}H NMR also indicate that the degree of stacking decreases as the electronegativity of the halo substituent decreases. Furthermore, steric bulk at the 2'-position of the sugar moiety is better accommodated in an A-form duplex than a B-
10   form duplex. Thus, a 2'-substituent on the 3'-terminus of a dinucleoside monophosphate is thought to exert a number of effects on the stacking conformation: steric repulsion, furanose puckering preference, electrostatic repulsion, hydrophobic attraction, and hydrogen bonding capabilities.
15   These substituent effects are thought to be determined by the molecular size, electronegativity, and hydrophobicity of the substituent. Melting temperatures of complementary strands is also increased with the 2'-substituted adenosine diphosphates. It is not clear whether the 3'-endo
20   preference of the conformation or the presence of the substituent is responsible for the increased binding. However, greater overlap of adjacent bases (stacking) can be achieved with the 3'-endo conformation.

      One synthetic 2'-modification that imparts increased
25   nuclease resistance and a very high binding affinity to nucleotides is the 2-methoxyethoxy (2'-MOE, 2'-OCH$_2$CH$_2$OCH$_3$) side chain (Baker *et al.*, *J. Biol. Chem.*, **1997**, *272*, 11944-12000). One of the immediate advantages of the 2'-MOE substitution is the improvement in binding affinity, which
30   is greater than many similar 2' modifications such as *O*-methyl, *O*-propyl, and *O*-aminopropyl. Oligonucleotides having the 2'-*O*-methoxyethyl substituent also have been shown to be antisense inhibitors of gene expression with promising features for *in vivo* use (Martin, P., *Helv. Chim.*
35   *Acta*, **1995**, *78*, 486-504; Altmann *et al.*, *Chimia*, **1996**, *50*, 168-176; Altmann *et al.*, *Biochem. Soc. Trans.*, **1996**, *24*,

630-637; and Altmann *et al., Nucleosides Nucleotides,* 1997,
16, 917-926). Relative to DNA, the oligonucleotides having
the 2'-MOE modification displayed improved RNA affinity and
higher nuclease resistance. Chimeric oligonucleotides
5   having 2'-MOE substituents in the wing nucleosides and an
internal region of deoxy-phosphorothioate nucleotides (also
termed a gapped oligonucleotide or gapmer) have shown
effective reduction in the growth of tumors in animal
models at low doses. 2'-MOE substituted oligonucleotides
10   have also shown outstanding promise as antisense agents in
several disease states. One such MOE substituted
oligonucleotide is presently being investigated in clinical
trials for the treatment of CMV retinitis.

15   **Chemistries Defined**
       Unless otherwise defined herein, alkyl means C_1-C_{12},
preferably C_1-C_8, and more preferably C_1-C_6, straight or
(where possible) branched chain aliphatic hydrocarbyl.
       Unless otherwise defined herein, heteroalkyl means C_1-
20   C_{12}, preferably C_1-C_8, and more preferably C_1-C_6, straight or
(where possible) branched chain aliphatic hydrocarbyl
containing at least one, and preferably about 1 to about 3,
hetero atoms in the chain, including the terminal portion
of the chain. Preferred heteroatoms include N, O and S.
25   Unless otherwise defined herein, cycloalkyl means C_3-
C_{12}, preferably C_3-C_8, and more preferably C_3-C_6, aliphatic
hydrocarbyl ring.
       Unless otherwise defined herein, alkenyl means C_2-C_{12},
preferably C_2-C_8, and more preferably C_2-C_6 alkenyl, which
30   may be straight or (where possible) branched hydrocarbyl
moiety, which contains at least one carbon-carbon double
bond.
       Unless otherwise defined herein, alkynyl means C_2-C_{12},
preferably C_2-C_8, and more preferably C_2-C_6 alkynyl, which
35   may be straight or (where possible) branched hydrocarbyl

moiety, which contains at least one carbon-carbon triple bond.

Unless otherwise defined herein, heterocycloalkyl means a ring moiety containing at least three ring members, at least one of which is carbon, and of which 1, 2 or three ring members are other than carbon. Preferably the number of carbon atoms varies from 1 to about 12, preferably 1 to about 6, and the total number of ring members varies from three to about 15, preferably from about 3 to about 8. Preferred ring heteroatoms are N, O and S. Preferred heterocycloalkyl groups include morpholino, thiomorpholino, piperidinyl, piperazinyl, homopiperidinyl, homopiperazinyl, homomorpholino, homothiomorpholino, pyrrolodinyl, tetrahydrooxazolyl, tetrahydroimidazolyl, tetrahydrothiazolyl, tetrahydroisoxazolyl, tetrahydropyrrazolyl, furanyl, pyranyl, and tetrahydroisothiazolyl.

Unless otherwise defined herein, aryl means any hydrocarbon ring structure containing at least one aryl ring. Preferred aryl rings have about 6 to about 20 ring carbons. Especially preferred aryl rings include phenyl, napthyl, anthracenyl, and phenanthrenyl.

Unless otherwise defined herein, hetaryl means a ring moiety containing at least one fully unsaturated ring, the ring consisting of carbon and non-carbon atoms. Preferably the ring system contains about 1 to about 4 rings. Preferably the number of carbon atoms varies from 1 to about 12, preferably 1 to about 6, and the total number of ring members varies from three to about 15, preferably from about 3 to about 8. Preferred ring heteroatoms are N, O and S. Preferred hetaryl moieties include pyrazolyl, thiophenyl, pyridyl, imidazolyl, tetrazolyl, pyridyl, pyrimidinyl, purinyl, quinazolinyl, quinoxalinyl, benzimidazolyl, benzothiophenyl, etc.

Unless otherwise defined herein, where a moiety is defined as a compound moiety, such as hetarylalkyl (hetaryl

and alkyl), aralkyl (aryl and alkyl), etc., each of the sub-moieties is as defined herein.

Unless otherwise defined herein, an electron withdrawing group is a group, such as the cyano or isocyanato group that draws electronic charge away from the carbon to which it is attached. Other electron withdrawing groups of note include those whose electronegativities exceed that of carbon, for example halogen, nitro, or phenyl substituted in the ortho- or para-position with one or more cyano, isothiocyanato, nitro or halo groups.

Unless otherwise defined herein, the terms halogen and halo have their ordinary meanings. Preferred halo (halogen) substituents are Cl, Br, and I. The aforementioned optional substituents are, unless otherwise herein defined, suitable substituents depending upon desired properties. Included are halogens (Cl, Br, I), alkyl, alkenyl, and alkynyl moieties, NO_2, NH_3 (substituted and unsubstituted), acid moieties (e.g. $-CO_2H$, $-OSO_3H_2$, etc.), heterocycloalkyl moieties, hetaryl moieties, aryl moieties, etc. In all the preceding formulae, the squiggle (~) indicates a bond to an oxygen or sulfur of the 5'-phosphate.

Phosphate protecting groups include those described in US Patents No. US 5,760,209, US 5,614,621, US 6,051,699, US 6,020,475, US 6,326,478, US 6,169,177, US 6,121,437, US 6,465,628 each of which is expressly incorporated herein by reference in its entirety.

The oligonucleotides in accordance with this invention (single stranded or double stranded) preferably comprise from about 8 to about 80 nucleotides, more preferably from about 12-50 nucleotides and most preferably from about 15 to 30 nucleotides. As is known in the art, a nucleotide is a base-sugar combination suitably bound to an adjacent nucleotide through a phosphodiester, phosphorothioate or other covalent linkage.

The oligonucleotides of the present invention also include variants in which a different base is present at

one or more of the nucleotide positions in the
oligonucleotide. For example, if the first nucleotide is an
adenosine, variants may be produced which contain
thymidine, guanosine or cytidine at this position. This
5   may be done at any of the positions of the oligonucleotide.
Thus, a 20-mer may comprise 60 variations (20 positions x 3
alternates at each position) in which the original
nucleotide is substituted with any of the three alternate
nucleotides. These oligonucleotides are then tested using
10   the methods described herein to determine their ability to
inhibit expression of superoxide dismutase-1, soluble mRNA.
     The antisense compounds used in accordance with this
invention may be conveniently and routinely made through
the well-known technique of solid phase synthesis.
15   Equipment for such synthesis is sold by several vendors
including, for example, Applied Biosystems (Foster City,
CA). Any other means for such synthesis known in the art
may additionally or alternatively be employed. It is well
known to use similar techniques to prepare oligonucleotides
20   such as the phosphorothioates and alkylated derivatives.
     The antisense compounds of the invention are
synthesized in vitro and do not include antisense
compositions of biological origin, or genetic vector
constructs designed to direct the in vivo synthesis of
25   antisense molecules.
     The compounds of the invention may also be admixed,
encapsulated, conjugated or otherwise associated with other
molecules, molecule structures or mixtures of compounds, as
for example, liposomes, receptor targeted molecules, oral,
30   rectal, topical or other formulations, for assisting in
uptake, distribution and/or absorption. Representative
United States patents that teach the preparation of such
uptake, distribution and/or absorption assisting
formulations include, but are not limited to, U.S.:
35   5,108,921; 5,354,844; 5,416,016; 5,459,127; 5,521,291;
5,543,158; 5,547,932; 5,583,020; 5,591,721; 4,426,330;
4,534,899; 5,013,556; 5,108,921; 5,213,804; 5,227,170;

5,264,221; 5,356,633; 5,395,619; 5,416,016; 5,417,978;
5,462,854; 5,469,854; 5,512,295; 5,527,528; 5,534,259;
5,543,152; 5,556,948; 5,580,575; and 5,595,756, each of
which is herein incorporated by reference.

5   The antisense compounds of the invention encompass any
pharmaceutically acceptable salts, esters, or salts of such
esters, or any other compound which, upon administration to
an animal including a human, is capable of providing
(directly or indirectly) the biologically active metabolite
10   or residue thereof. Accordingly, for example, the
disclosure is also drawn to prodrugs and pharmaceutically
acceptable salts of the compounds of the invention,
pharmaceutically acceptable salts of such prodrugs, and
other bioequivalents.

15   The term "prodrug" indicates a therapeutic agent that
is prepared in an inactive form that is converted to an
active form (i.e., drug) within the body or cells thereof
by the action of endogenous enzymes or other chemicals
and/or conditions. In particular, prodrug versions of the
20   oligonucleotides of the invention are prepared as SATE
[(S-acetyl-2-thioethyl) phosphate] derivatives according to
the methods disclosed in WO 93/24510 to Gosselin et al.,
published December 9, 1993 or in WO 94/26764 and U.S.
5,770,713 to Imbach et al.

25   The term "pharmaceutically acceptable salts" refers to
physiologically and pharmaceutically acceptable salts of
the compounds of the invention: i.e., salts that retain the
desired biological activity of the parent compound and do
not impart undesired toxicological effects thereto.

30   Pharmaceutically acceptable base addition salts are
formed with metals or amines, such as alkali and alkaline
earth metals or organic amines. Examples of metals used as
cations are sodium, potassium, magnesium, calcium, and the
like. Examples of suitable amines are
35 N,N'-dibenzylethylenediamine, chloroprocaine, choline,
diethanolamine, dicyclohexylamine, ethylenediamine,
N-methylglucamine, and procaine (see, for example, Berge et

al., "Pharmaceutical Salts," *J. of Pharma Sci.*, **1977**, *66*,
1-19). The base addition salts of said acidic compounds
are prepared by contacting the free acid form with a
sufficient amount of the desired base to produce the salt
5   in the conventional manner. The free acid form may be
regenerated by contacting the salt form with an acid and
isolating the free acid in the conventional manner. The
free acid forms differ from their respective salt forms
somewhat in certain physical properties such as solubility
10 in polar solvents, but otherwise the salts are equivalent
to their respective free acid for purposes of the present
invention. As used herein, a "pharmaceutical addition
salt" includes a pharmaceutically acceptable salt of an
acid form of one of the components of the compositions of
15 the invention. These include organic or inorganic acid
salts of the amines. Preferred acid salts are the
hydrochlorides, acetates, salicylates, nitrates and
phosphates. Other suitable pharmaceutically acceptable
salts are well known to those skilled in the art and
20 include basic salts of a variety of inorganic and organic
acids, such as, for example, with inorganic acids, such as
for example hydrochloric acid, hydrobromic acid, sulfuric
acid or phosphoric acid; with organic carboxylic, sulfonic,
sulfo or phospho acids or N-substituted sulfamic acids, for
25 example acetic acid, propionic acid, glycolic acid,
succinic acid, maleic acid, hydroxymaleic acid,
methylmaleic acid, fumaric acid, malic acid, tartaric acid,
lactic acid, oxalic acid, gluconic acid, glucaric acid,
glucuronic acid, citric acid, benzoic acid, cinnamic acid,
30 mandelic acid, salicylic acid, 4-aminosalicylic acid,
2-phenoxybenzoic acid, 2-acetoxybenzoic acid, embonic acid,
nicotinic acid or isonicotinic acid; and with amino acids,
such as the 20 alpha-amino acids involved in the synthesis
of proteins in nature, for example glutamic acid or
35 aspartic acid, and also with phenylacetic acid,
methanesulfonic acid, ethanesulfonic acid,
2-hydroxyethanesulfonic acid, ethane-1,2-disulfonic acid,

benzenesulfonic acid, 4-methylbenzenesulfonic acid,
naphthalene-2-sulfonic acid, naphthalene-1,5-disulfonic
acid, 2- or 3-phosphoglycerate, glucose-6-phosphate,
N-cyclohexylsulfamic acid (with the formation of
5   cyclamates), or with other acid organic compounds, such as
ascorbic acid. Pharmaceutically acceptable salts of
compounds may also be prepared with a pharmaceutically
acceptable cation. Suitable pharmaceutically acceptable
cations are well known to those skilled in the art and
10   include alkaline, alkaline earth, ammonium and quaternary
ammonium cations. Carbonates or hydrogen carbonates are
also possible.

      For oligonucleotides, preferred examples of
pharmaceutically acceptable salts include but are not
15   limited to (a) salts formed with cations such as sodium,
potassium, ammonium, magnesium, calcium, polyamines such as
spermine and spermidine, etc.; (b) acid addition salts
formed with inorganic acids, for example hydrochloric acid,
hydrobromic acid, sulfuric acid, phosphoric acid, nitric
20   acid and the like; (c) salts formed with organic acids
such as, for example, acetic acid, oxalic acid, tartaric
acid, succinic acid, maleic acid, fumaric acid, gluconic
acid, citric acid, malic acid, ascorbic acid, benzoic acid,
tannic acid, palmitic acid, alginic acid, polyglutamic
25   acid, naphthalenesulfonic acid, methanesulfonic acid,
p-toluenesulfonic acid, naphthalenedisulfonic acid,
polygalacturonic acid, and the like; and (d) salts formed
from elemental anions such as chlorine, bromine, and
iodine.
30   The antisense compounds of the present invention can
be utilized for diagnostics, therapeutics, prophylaxis and
as research reagents and kits. For therapeutics, an
animal, preferably a human, suspected of having a disease
or disorder which can be treated by modulating the
35   expression of superoxide dismutase 1, soluble is treated by
administering antisense compounds in accordance with this
invention. The compounds of the invention can be utilized

in pharmaceutical compositions by adding an effective amount of an antisense compound to a suitable pharmaceutically acceptable diluent or carrier. Use of the antisense compounds and methods of the invention may also

5   be useful prophylactically, e.g., to prevent or delay infection, inflammation or tumor formation, for example.

The antisense compounds of the invention are useful for research and diagnostics, because these compounds hybridize to nucleic acids encoding superoxide dismutase 1,

10 soluble, enabling sandwich and other assays to easily be constructed to exploit this fact. Hybridization of the antisense oligonucleotides of the invention with a nucleic acid encoding superoxide dismutase 1, soluble can be detected by means known in the art. Such means may include

15 conjugation of an enzyme to the oligonucleotide, radiolabelling of the oligonucleotide or any other suitable detection means. Kits using such detection means for detecting the level of superoxide dismutase 1, soluble in a sample may also be prepared.

20   The present invention also includes pharmaceutical compositions and formulations which include the antisense compounds of the invention. The pharmaceutical compositions of the present invention may be administered in a number of ways depending upon whether local or

25 systemic treatment is desired and upon the area to be treated. Administration may be topical (including ophthalmic and to mucous membranes including vaginal and rectal delivery), pulmonary, e.g., by inhalation or insufflation of powders or aerosols, including by

30 nebulizer; intratracheal, intranasal, epidermal and transdermal), oral or parenteral. Parenteral administration includes intravenous, intraarterial, subcutaneous, intraperitoneal or intramuscular injection or infusion; or intracranial, e.g., intrathecal or

35 intraventricular, administration. Oligonucleotides with at least one 2'-O-methoxyethyl modification are believed to be particularly useful for oral administration.

Pharmaceutical compositions and formulations for topical administration may include transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical

5 carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable. Coated condoms, gloves and the like may also be useful. Preferred topical formulations include those in which the oligonucleotides of the invention are in admixture with a topical delivery

10 agent such as lipids, liposomes, fatty acids, fatty acid esters, steroids, chelating agents and surfactants. Preferred lipids and liposomes include neutral (e.g. dioleoylphosphatidyl DOPE ethanolamine, dimyristoylphosphatidyl choline DMPC,

15 distearolyphosphatidyl choline) negative (e.g. dimyristoylphosphatidyl glycerol DMPG) and cationic (e.g. dioleoyltetramethylaminopropyl DOTAP and dioleoylphosphatidyl ethanolamine DOTMA). Oligonucleotides of the invention may be encapsulated within liposomes or

20 may form complexes thereto, in particular to cationic liposomes. Alternatively, oligonucleotides may be complexed to lipids, in particular to cationic lipids. Preferred fatty acids and esters include but are not limited arachidonic acid, oleic acid, eicosanoic acid,

25 lauric acid, caprylic acid, capric acid, myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprate, tricaprate, monoolein, dilaurin, glyceryl 1-monocaprate, 1-dodecylazacycloheptan-2-one, an acylcarnitine, an acylcholine, or a C_{1-10} alkyl ester (e.g.

30 isopropylmyristate IPM), monoglyceride, diglyceride or pharmaceutically acceptable salt thereof. Topical formulations are described in detail in United States patent application 09/315,298 filed on May 20, 1999 which is incorporated herein by reference in its entirety.

35   Compositions and formulations for oral administration include powders or granules, microparticulates, nanoparticulates, suspensions or solutions in water or non-

aqueous media, capsules, gel capsules, sachets, tablets or
minitablets. Thickeners, flavoring agents, diluents,
emulsifiers, dispersing aids or binders may be desirable.
Preferred oral formulations are those in which

5   oligonucleotides of the invention are administered in
conjunction with one or more penetration enhancers
surfactants and chelators. Preferred surfactants include
fatty acids and/or esters or salts thereof, bile acids
and/or salts thereof. Prefered bile acids/salts include

10 chenodeoxycholic acid (CDCA) and ursodeoxychenodeoxycholic
acid (UDCA), cholic acid, dehydrocholic acid, deoxycholic
acid, glucholic acid, glycholic acid, glycodeoxycholic
acid, taurocholic acid, taurodeoxycholic acid, sodium
tauro-24,25-dihydro-fusidate, sodium glycodihydrofusidate,.

15 Prefered fatty acids include arachidonic acid, undecanoic
acid, oleic acid, lauric acid, caprylic acid, capric acid,
myristic acid, palmitic acid, stearic acid, linoleic acid,
linolenic acid, dicaprate, tricaprate, monoolein, dilaurin,
glyceryl 1-monocaprate, 1-dodecylazacycloheptan-2-one, an

20 acylcarnitine, an acylcholine, or a monoglyceride, a
diglyceride or a pharmaceutically acceptable salt thereof
(e.g. sodium). Also prefered are combinations of
penetration enhancers, for example, fatty acids/salts in
combination with bile acids/salts. A particularly prefered

25 combination is the sodium salt of lauric acid, capric acid
and UDCA. Further penetration enhancers include
polyoxyethylene-9-lauryl ether, polyoxyethylene-20-cetyl
ether. Oligonucleotides of the invention may be delivered
orally in granular form including sprayed dried particles,

30 or complexed to form micro or nanoparticles.
Oligonucleotide complexing agents include
poly-amino acids; polyimines; polyacrylates;
polyalkylacrylates, polyoxethanes, polyalkylcyanoacrylates;
cationized gelatins, albumins, starches, acrylates,

35 polyethyleneglycols (PEG) and starches;
polyalkylcyanoacrylates; DEAE-derivatized polyimines,
pollulans, celluloses and starches. Particularly preferred

complexing agents include chitosan, N-trimethylchitosan, poly-L-lysine, polyhistidine, polyornithine, polyspermines, protamine, polyvinylpyridine, polythiodiethylamino-methylethylene P(TDAE), polyaminostyrene (e.g. p-amino),
5 poly(methylcyanoacrylate), poly(ethylcyanoacrylate), poly(butylcyanoacrylate), poly(isobutylcyanoacrylate), poly(isohexylcynaoacrylate), DEAE-methacrylate, DEAE-hexylacrylate, DEAE-acrylamide, DEAE-albumin and DEAE-dextran, polymethylacrylate, polyhexylacrylate, poly(D,L-
10 lactic acid), poly(DL-lactic-co-glycolic acid (PLGA), alginate, and polyethyleneglycol (PEG). Oral formulations for oligonucleotides and their preparation are described in detail in United States applications 08/886,829 (filed July 1, 1997), 09/108,673 (filed July 1, 1998), 09/256,515
15 (filed February 23, 1999), 09/082,624 (filed May 21, 1998) and 09/315,298 (filed May 20, 1999) each of which is incorporated herein by reference in their entirety.

Compositions and formulations for parenteral, intrathecal or intraventricular administration may include
20 sterile aqueous solutions which may also contain buffers, diluents and other suitable additives such as, but not limited to, penetration enhancers, carrier compounds and other pharmaceutically acceptable carriers or excipients.

Pharmaceutical compositions of the present invention
25 include, but are not limited to, solutions, emulsions, and liposome-containing formulations. These compositions may be generated from a variety of components that include, but are not limited to, preformed liquids, self-emulsifying solids and self-emulsifying semisolids.
30 The pharmaceutical formulations of the present invention, which may conveniently be presented in unit dosage form, may be prepared according to conventional techniques well known in the pharmaceutical industry. Such techniques include the step of bringing into association
35 the active ingredients with the pharmaceutical carrier(s) or excipient(s). In general the formulations are prepared by uniformly and intimately bringing into association the

active ingredients with liquid carriers or finely divided
solid carriers or both, and then, if necessary, shaping the
product.

5   The compositions of the present invention may be
formulated into any of many possible dosage forms such as,
but not limited to, tablets, capsules, gel capsules, liquid
syrups, soft gels, suppositories, and enemas. The
compositions of the present invention may also be
formulated as suspensions in aqueous, non-aqueous or mixed
10   media. Aqueous suspensions may further contain substances
which increase the viscosity of the suspension including,
for example, sodium carboxymethylcellulose, sorbitol and/or
dextran. The suspension may also contain stabilizers.

        In one embodiment of the present invention the
15   pharmaceutical compositions may be formulated and used as
foams. Pharmaceutical foams include formulations such as,
but not limited to, emulsions, microemulsions, creams,
jellies and liposomes. While basically similar in nature
these formulations vary in the components and the
20   consistency of the final product. The preparation of such
compositions and formulations is generally known to those
skilled in the pharmaceutical and formulation arts and may
be applied to the formulation of the compositions of the
present invention.
25

Emulsions

        The compositions of the present invention may be
prepared and formulated as emulsions. Emulsions are
typically heterogenous systems of one liquid dispersed in
30   another in the form of droplets usually exceeding 0.1 μm in
diameter. (Idson, in *Pharmaceutical Dosage Forms*,
Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker,
Inc., New York, N.Y., volume 1, p. 199; Rosoff, in
Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker
35   (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., Volume
1, p. 245; Block in *Pharmaceutical Dosage Forms*, Lieberman,

Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 2, p. 335; Higuchi *et al.*, in *Remington's Pharmaceutical Sciences*, Mack Publishing Co., Easton, PA, **1985**, p. 301). Emulsions are often biphasic
5   systems comprising of two immiscible liquid phases intimately mixed and dispersed with each other. In general, emulsions may be either water-in-oil (w/o) or of the oil-in-water (o/w) variety. When an aqueous phase is finely divided into and dispersed as minute droplets into a
10   bulk oily phase the resulting composition is called a water-in-oil (w/o) emulsion. Alternatively, when an oily phase is finely divided into and dispersed as minute droplets into a bulk aqueous phase the resulting composition is called an oil-in-water (o/w) emulsion.
15   Emulsions may contain additional components in addition to the dispersed phases and the active drug which may be present as a solution in either the aqueous phase, oily phase or itself as a separate phase. Pharmaceutical excipients such as emulsifiers, stabilizers, dyes, and
20   anti-oxidants may also be present in emulsions as needed. Pharmaceutical emulsions may also be multiple emulsions that are comprised of more than two phases such as, for example, in the case of oil-in-water-in-oil (o/w/o) and water-in-oil-in-water (w/o/w) emulsions. Such complex
25   formulations often provide certain advantages that simple binary emulsions do not. Multiple emulsions in which individual oil droplets of an o/w emulsion enclose small water droplets constitute a w/o/w emulsion. Likewise a system of oil droplets enclosed in globules of water
30   stabilized in an oily continuous provides an o/w/o emulsion.

Emulsions are characterized by little or no thermodynamic stability. Often, the dispersed or discontinuous phase of the emulsion is well dispersed into
35   the external or continuous phase and maintained in this form through the means of emulsifiers or the viscosity of

the formulation. Either of the phases of the emulsion may
be a semisolid or a solid, as is the case of emulsion-style
ointment bases and creams. Other means of stabilizing
emulsions entail the use of emulsifiers that may be
5 incorporated into either phase of the emulsion.
Emulsifiers may broadly be classified into four categories:
synthetic surfactants, naturally occurring emulsifiers,
absorption bases, and finely dispersed solids (Idson, in
Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker
10 (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume
1, p. 199).

Synthetic surfactants, also known as surface active
agents, have found wide applicability in the formulation of
emulsions and have been reviewed in the literature (Rieger,
15 in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and
Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y.,
volume 1, p. 285; Idson, in *Pharmaceutical Dosage Forms*,
Lieberman, Rieger and Banker (Eds.), Marcel Dekker, Inc.,
New York, N.Y., **1988**, volume 1, p. 199). Surfactants are
20 typically amphiphilic and comprise a hydrophilic and a
hydrophobic portion. The ratio of the hydrophilic to the
hydrophobic nature of the surfactant has been termed the
hydrophile/lipophile balance (HLB) and is a valuable tool
in categorizing and selecting surfactants in the
25 preparation of formulations. Surfactants may be classified
into different classes based on the nature of the
hydrophilic group: nonionic, anionic, cationic and
amphoteric (Rieger, in *Pharmaceutical Dosage Forms*,
Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker,
30 Inc., New York, N.Y., volume 1, p. 285).

Naturally occurring emulsifiers used in emulsion
formulations include lanolin, beeswax, phosphatides,
lecithin and acacia. Absorption bases possess hydrophilic
properties such that they can soak up water to form w/o
35 emulsions yet retain their semisolid consistencies, such as

anhydrous lanolin and hydrophilic petrolatum. Finely
divided solids have also been used as good emulsifiers
especially in combination with surfactants and in viscous
preparations. These include polar inorganic solids, such

5   as heavy metal hydroxides, nonswelling clays such as
bentonite, attapulgite, hectorite, kaolin, montmorillonite,
colloidal aluminum silicate and colloidal magnesium
aluminum silicate, pigments and nonpolar solids such as
carbon or glyceryl tristearate.

10   A large variety of non-emulsifying materials are also
included in emulsion formulations and contribute to the
properties of emulsions. These include fats, oils, waxes,
fatty acids, fatty alcohols, fatty esters, humectants,
hydrophilic colloids, preservatives and antioxidants

15 (Block, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger
and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York,
N.Y., volume 1, p. 335; Idson, in *Pharmaceutical Dosage
Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel
Dekker, Inc., New York, N.Y., volume 1, p. 199).

20   Hydrophilic colloids or hydrocolloids include
naturally occurring gums and synthetic polymers such as
polysaccharides (for example, acacia, agar, alginic acid,
carrageenan, guar gum, karaya gum, and tragacanth),
cellulose derivatives (for example, carboxymethylcellulose

25 and carboxypropylcellulose), and synthetic polymers (for
example, carbomers, cellulose ethers, and carboxyvinyl
polymers). These disperse or swell in water to form
colloidal solutions that stabilize emulsions by forming
strong interfacial films around the dispersed-phase

30 droplets and by increasing the viscosity of the external
phase.

        Since emulsions often contain a number of ingredients
such as carbohydrates, proteins, sterols and phosphatides
that may readily support the growth of microbes, these

35 formulations often incorporate preservatives. Commonly
used preservatives included in emulsion formulations

include methyl paraben, propyl paraben, quaternary ammonium salts, benzalkonium chloride, esters of p-hydroxybenzoic acid, and boric acid. Antioxidants are also commonly added to emulsion formulations to prevent deterioration of the

5   formulation. Antioxidants used may be free radical scavengers such as tocopherols, alkyl gallates, butylated hydroxyanisole, butylated hydroxytoluene, or reducing agents such as ascorbic acid and sodium metabisulfite, and antioxidant synergists such as citric acid, tartaric acid,

10   and lecithin.

The application of emulsion formulations via dermatological, oral and parenteral routes and methods for their manufacture have been reviewed in the literature (Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger

15   and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199). Emulsion formulations for oral delivery have been very widely used because of reasons of ease of formulation, efficacy from an absorption and bioavailability standpoint. (Rosoff, in *Pharmaceutical*

20   *Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245; Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199). Mineral-oil base laxatives, oil-

25   soluble vitamins and high fat nutritive preparations are among the materials that have commonly been administered orally as o/w emulsions.

In one embodiment of the present invention, the compositions of oligonucleotides and nucleic acids are

30   formulated as microemulsions. A microemulsion may be defined as a system of water, oil and amphiphile which is a single optically isotropic and thermodynamically stable liquid solution (Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker,

35   Inc., New York, N.Y., volume 1, p. 245). Typically

microemulsions are systems that are prepared by first
dispersing an oil in an aqueous surfactant solution and
then adding a sufficient amount of a fourth component,
generally an intermediate chain-length alcohol to form a

5   transparent system. Therefore, microemulsions have also
been described as thermodynamically stable, isotropically
clear dispersions of two immiscible liquids that are
stabilized by interfacial films of surface-active molecules
(Leung and Shah, *in: Controlled Release of Drugs: Polymers*

10   *and Aggregate Systems*, Rosoff, M., Ed., **1989**, VCH
Publishers, New York, pages 185-215). Microemulsions
commonly are prepared via a combination of three to five
components that include oil, water, surfactant,
cosurfactant and electrolyte. Whether the microemulsion is

15   of the water-in-oil (w/o) or an oil-in-water (o/w) type is
dependent on the properties of the oil and surfactant used
and on the structure and geometric packing of the polar
heads and hydrocarbon tails of the surfactant molecules
(Schott, in *Remington's Pharmaceutical Sciences*, Mack

20   Publishing Co., Easton, PA, **1985**, p. 271).

The phenomenological approach utilizing phase diagrams
has been extensively studied and has yielded a
comprehensive knowledge, to one skilled in the art, of how
to formulate microemulsions (Rosoff, in *Pharmaceutical*

25   *Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**,
Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245;
Block, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger
and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York,
N.Y., volume 1, p. 335). Compared to conventional

30   emulsions, microemulsions offer the advantage of
solubilizing water-insoluble drugs in a formulation of
thermodynamically stable droplets that are formed
spontaneously.

Surfactants used in the preparation of microemulsions

35   include, but are not limited to, ionic surfactants, non-

ionic surfactants, Brij 96, polyoxyethylene oleyl ethers, polyglycerol fatty acid esters, tetraglycerol monolaurate (ML310), tetraglycerol monooleate (MO310), hexaglycerol monooleate (PO310), hexaglycerol pentaoleate (PO500),

5   decaglycerol monocaprate (MCA750), decaglycerol monooleate (MO750), decaglycerol sequioleate (SO750), decaglycerol decaoleate (DAO750), alone or in combination with cosurfactants. The cosurfactant, usually a short-chain alcohol such as ethanol, 1-propanol, and 1-butanol, serves

10 to increase the interfacial fluidity by penetrating into the surfactant film and consequently creating a disordered film because of the void space generated among surfactant molecules. Microemulsions may, however, be prepared without the use of cosurfactants and alcohol-free self-

15 emulsifying microemulsion systems are known in the art. The aqueous phase may typically be, but is not limited to, water, an aqueous solution of the drug, glycerol, PEG300, PEG400, polyglycerols, propylene glycols, and derivatives of ethylene glycol. The oil phase may include, but is not

20 limited to, materials such as Captex 300, Captex 355, Capmul MCM, fatty acid esters, medium chain (C8-C12) mono, di, and tri-glycerides, polyoxyethylated glyceryl fatty acid esters, fatty alcohols, polyglycolized glycerides, saturated polyglycolized C8-C10 glycerides, vegetable oils

25 and silicone oil.

        Microemulsions are particularly of interest from the standpoint of drug solubilization and the enhanced absorption of drugs. Lipid based microemulsions (both o/w and w/o) have been proposed to enhance the oral

30 bioavailability of drugs, including peptides (Constantinides et al., *Pharmaceutical Research*, **1994**, *11*, 1385-1390; Ritschel, *Meth. Find. Exp. Clin. Pharmacol.*, **1993**, *13*, 205). Microemulsions afford advantages of improved drug solubilization, protection of drug from

35 enzymatic hydrolysis, possible enhancement of drug absorption due to surfactant-induced alterations in

membrane fluidity and permeability, ease of preparation, ease of oral administration over solid dosage forms, improved clinical potency, and decreased toxicity (Constantinides et al., *Pharmaceutical Research*, **1994**, *11*,

5 1385; Ho *et al.*, *J. Pharm. Sci.*, **1996**, *85*, 138-143). Often microemulsions may form spontaneously when their components are brought together at ambient temperature. This may be particularly advantageous when formulating thermolabile drugs, peptides or oligonucleotides. Microemulsions have

10 also been effective in the transdermal delivery of active components in both cosmetic and pharmaceutical applications. It is expected that the microemulsion compositions and formulations of the present invention will facilitate the increased systemic absorption of

15 oligonucleotides and nucleic acids from the gastrointestinal tract, as well as improve the local cellular uptake of oligonucleotides and nucleic acids within the gastrointestinal tract, vagina, buccal cavity and other areas of administration.

20   Microemulsions of the present invention may also contain additional components and additives such as sorbitan monostearate (Grill 3), Labrasol, and penetration enhancers to improve the properties of the formulation and to enhance the absorption of the oligonucleotides and

25 nucleic acids of the present invention. Penetration enhancers used in the microemulsions of the present invention may be classified as belonging to one of five broad categories - surfactants, fatty acids, bile salts, chelating agents, and non-chelating non-surfactants (Lee *et*

30 *al.*, *Critical Reviews in Therapeutic Drug Carrier Systems*, **1991**, p. 92). Each of these classes has been discussed above.

Liposomes

35   There are many organized surfactant structures besides microemulsions that have been studied and used for the

formulation of drugs. These include monolayers, micelles, bilayers and vesicles. Vesicles, such as liposomes, have attracted great interest because of their specificity and the duration of action they offer from the standpoint of

5   drug delivery. As used in the present invention, the term "liposome" means a vesicle composed of amphiphilic lipids arranged in a spherical bilayer or bilayers.

Liposomes are unilamellar or multilamellar vesicles which have a membrane formed from a lipophilic material and

10   an aqueous interior. The aqueous portion contains the composition to be delivered. Cationic liposomes possess the advantage of being able to fuse to the cell wall. Non-cationic liposomes, although not able to fuse as efficiently with the cell wall, are taken up by macrophages

15   *in vivo*.

In order to cross intact mammalian skin, lipid vesicles must pass through a series of fine pores, each with a diameter less than 50 nm, under the influence of a suitable transdermal gradient. Therefore, it is desirable

20   to use a liposome which is highly deformable and able to pass through such fine pores.

Further advantages of liposomes include; liposomes obtained from natural phospholipids are biocompatible and biodegradable; liposomes can incorporate a wide range of

25   water and lipid soluble drugs; liposomes can protect encapsulated drugs in their internal compartments from metabolism and degradation (Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245).

30   Important considerations in the preparation of liposome formulations are the lipid surface charge, vesicle size and the aqueous volume of the liposomes.

Liposomes are useful for the transfer and delivery of active ingredients to the site of action. Because the

35   liposomal membrane is structurally similar to biological membranes, when liposomes are applied to a tissue, the

liposomes start to merge with the cellular membranes. As the merging of the liposome and cell progresses, the liposomal contents are emptied into the cell where the active agent may act.

5   Liposomal formulations have been the focus of extensive investigation as the mode of delivery for many drugs. There is growing evidence that for topical administration, liposomes present several advantages over other formulations. Such advantages include reduced side-

10   effects related to high systemic absorption of the administered drug, increased accumulation of the administered drug at the desired target, and the ability to administer a wide variety of drugs, both hydrophilic and hydrophobic, into the skin.

15   Several reports have detailed the ability of liposomes to deliver agents including high-molecular weight DNA into the skin. Compounds including analgesics, antibodies, hormones and high-molecular weight DNAs have been administered to the skin. The majority of applications

20   resulted in the targeting of the upper epidermis.

Liposomes fall into two broad classes. Cationic liposomes are positively charged liposomes which interact with the negatively charged DNA molecules to form a stable complex. The positively charged DNA/liposome complex binds

25   to the negatively charged cell surface and is internalized in an endosome. Due to the acidic pH within the endosome, the liposomes are ruptured, releasing their contents into the cell cytoplasm (Wang *et al.*, *Biochem. Biophys. Res. Commun.*, *1987, 147*, 980-985).

30   Liposomes which are pH-sensitive or negatively-charged, entrap DNA rather than complex with it. Since both the DNA and the lipid are similarly charged, repulsion rather than complex formation occurs. Nevertheless, some DNA is entrapped within the aqueous

35   interior of these liposomes. pH-sensitive liposomes have been used to deliver DNA encoding the thymidine kinase gene to cell monolayers in culture. Expression of the exogenous

gene was detected in the target cells (Zhou *et al.*, *Journal of Controlled Release*, **1992**, *19*, 269-274).

One major type of liposomal composition includes phospholipids other than naturally-derived phosphatidylcholine. Neutral liposome compositions, for example, can be formed from dimyristoyl phosphatidylcholine (DMPC) or dipalmitoyl phosphatidylcholine (DPPC). Anionic liposome compositions generally are formed from dimyristoyl phosphatidylglycerol, while anionic fusogenic liposomes are formed primarily from dioleoyl phosphatidylethanolamine (DOPE). Another type of liposomal composition is formed from phosphatidylcholine (PC) such as, for example, soybean PC, and egg PC. Another type is formed from mixtures of phospholipid and/or phosphatidylcholine and/or cholesterol.

Several studies have assessed the topical delivery of liposomal drug formulations to the skin. Application of liposomes containing interferon to guinea pig skin resulted in a reduction of skin herpes sores while delivery of interferon via other means (*e.g.* as a solution or as an emulsion) were ineffective (Weiner *et al.*, *Journal of Drug Targeting*, **1992**, *2*, 405-410). Further, an additional study tested the efficacy of interferon administered as part of a liposomal formulation to the administration of interferon using an aqueous system, and concluded that the liposomal formulation was superior to aqueous administration (du Plessis *et al.*, *Antiviral Research*, **1992**, *18*, 259-265).

Non-ionic liposomal systems have also been examined to determine their utility in the delivery of drugs to the skin, in particular systems comprising non-ionic surfactant and cholesterol. Non-ionic liposomal formulations comprising Novasome™ I (glyceryl dilaurate/cholesterol/polyoxyethylene-10-stearyl ether) and Novasome™ II (glyceryl distearate/ cholesterol/polyoxyethylene-10-stearyl ether) were used to deliver cyclosporin-A into the dermis of mouse skin.

Results indicated that such non-ionic liposomal systems
were effective in facilitating the deposition of
cyclosporin-A into different layers of the skin (Hu et al.
S.T.P.Pharma. Sci., **1994**, 4, 6, 466).

5   Liposomes also include "sterically stabilized"
liposomes, a term which, as used herein, refers to
liposomes comprising one or more specialized lipids that,
when incorporated into liposomes, result in enhanced
circulation lifetimes relative to liposomes lacking such
10   specialized lipids. Examples of sterically stabilized
liposomes are those in which part of the vesicle-forming
lipid portion of the liposome (A) comprises one or more
glycolipids, such as monosialoganglioside G_{M1}, or (B) is
derivatized with one or more hydrophilic polymers, such as
15   a polyethylene glycol (PEG) moiety. While not wishing to
be bound by any particular theory, it is thought in the art
that, at least for sterically stabilized liposomes
containing gangliosides, sphingomyelin, or PEG-derivatized
lipids, the enhanced circulation half-life of these
20   sterically stabilized liposomes derives from a reduced
uptake into cells of the reticuloendothelial system (RES)
(Allen *et al.*, *FEBS Letters*, **1987**, *223*, 42; Wu *et al.*,
Cancer Research, **1993**, *53*, 3765). Various liposomes
comprising one or more glycolipids are known in the art.
25   Papahadjopoulos *et al.* (*Ann. N.Y. Acad. Sci.*, **1987**, *507*,
64) reported the ability of monosialoganglioside G_{M1},
galactocerebroside sulfate and phosphatidylinositol to
improve blood half-lives of liposomes. These findings were
expounded upon by Gabizon *et al.* (*Proc. Natl. Acad. Sci.*
30   *U.S.A.*, **1988**, *85*, 6949). U.S. Patent No. 4,837,028 and WO
88/04924, both to Allen *et al.*, disclose liposomes
comprising (1) sphingomyelin and (2) the ganglioside G_{M1} or a
galactocerebroside sulfate ester. U.S. Patent No.
5,543,152 (Webb *et al.*) discloses liposomes comprising
35   sphingomyelin. Liposomes comprising 1,2-*sn-*

dimyristoylphosphatidylcholine are disclosed in WO 97/13499
(Lim *et al.*).

    Many liposomes comprising lipids derivatized with one
or more hydrophilic polymers, and methods of preparation

5   thereof, are known in the art. Sunamoto *et al.* (*Bull.
Chem. Soc. Jpn.*, **1980,** *53,* 2778) described liposomes
comprising a nonionic detergent, $2C_{12}15G$, that contains a
PEG moiety. Illum *et al.* (*FEBS Lett.*, **1984,** *167,* 79) noted
that hydrophilic coating of polystyrene particles with

10 polymeric glycols results in significantly enhanced blood
half-lives. Synthetic phospholipids modified by the
attachment of carboxylic groups of polyalkylene glycols
(*e.g.*, PEG) are described by Sears (U.S. Patent Nos.
4,426,330 and 4,534,899). Klibanov *et al.* (*FEBS Lett.*,

15 **1990,** *268,* 235) described experiments demonstrating that
liposomes comprising phosphatidylethanolamine (PE)
derivatized with PEG or PEG stearate have significant
increases in blood circulation half-lives. Blume *et al.*
(*Biochimica et Biophysica Acta,* **1990,** *1029,* 91) extended

20 such observations to other PEG-derivatized phospholipids,
e.g., DSPE-PEG, formed from the combination of
distearoylphosphatidylethanolamine (DSPE) and PEG.
Liposomes having covalently bound PEG moieties on their
external surface are described in European Patent No. EP 0

25 445 131 B1 and WO 90/04384 to Fisher. Liposome
compositions containing 1-20 mole percent of PE derivatized
with PEG, and methods of use thereof, are described by
Woodle *et al.* (U.S. Patent Nos. 5,013,556 and 5,356,633)
and Martin *et al.* (U.S. Patent No. 5,213,804 and European

30 Patent No. EP 0 496 813 B1). Liposomes comprising a number
of other lipid-polymer conjugates are disclosed in WO
91/05545 and U.S. Patent No. 5,225,212 (both to Martin *et
al.*) and in WO 94/20073 (Zalipsky *et al.*) Liposomes
comprising PEG-modified ceramide lipids are described in WO

96/10391 (Choi *et al.*). U.S. Patent Nos. 5,540,935

(Miyazaki *et al.*) and 5,556,948 (Tagawa *et al.*) describe

PEG-containing liposomes that can be further derivatized

with functional moieties on their surfaces.

5   A limited number of liposomes comprising nucleic acids

are known in the art. WO 96/40062 to Thierry *et al.*

discloses methods for encapsulating high molecular weight

nucleic acids in liposomes. U.S. Patent No. 5,264,221 to

Tagawa *et al.* discloses protein-bonded liposomes and

10   asserts that the contents of such liposomes may include an

antisense RNA. U.S. Patent No. 5,665,710 to Rahman *et al.*

describes certain methods of encapsulating

oligodeoxynucleotides in liposomes. WO 97/04787 to Love *et*

al. discloses liposomes comprising antisense

15   oligonucleotides targeted to the raf gene.

Transfersomes are yet another type of liposomes, and

are highly deformable lipid aggregates which are attractive

candidates for drug delivery vehicles. Transfersomes may

be described as lipid droplets which are so highly

20   deformable that they are easily able to penetrate through

pores which are smaller than the droplet. Transfersomes

are adaptable to the environment in which they are used,

e.g. they are self-optimizing (adaptive to the shape of

pores in the skin), self-repairing, frequently reach their

25   targets without fragmenting, and often self-loading. To

make transfersomes it is possible to add surface edge-

activators, usually surfactants, to a standard liposomal

composition. Transfersomes have been used to deliver serum

albumin to the skin. The transfersome-mediated delivery of

30   serum albumin has been shown to be as effective as

subcutaneous injection of a solution containing serum

albumin.

Surfactants find wide application in formulations such

as emulsions (including microemulsions) and liposomes. The

35   most common way of classifying and ranking the properties

of the many different types of surfactants, both natural and synthetic, is by the use of the hydrophile/lipophile balance (HLB). The nature of the hydrophilic group (also known as the "head") provides the most useful means for

5   categorizing the different surfactants used in formulations (Rieger, in *Pharmaceutical Dosage Forms*, Marcel Dekker, Inc., New York, NY, **1988**, p. 285).

If the surfactant molecule is not ionized, it is classified as a nonionic surfactant. Nonionic surfactants

10 find wide application in pharmaceutical and cosmetic products and are usable over a wide range of pH values. In general their HLB values range from 2 to about 18 depending on their structure. Nonionic surfactants include nonionic esters such as ethylene glycol esters, propylene glycol

15 esters, glyceryl esters, polyglyceryl esters, sorbitan esters, sucrose esters, and ethoxylated esters. Nonionic alkanolamides and ethers such as fatty alcohol ethoxylates, propoxylated alcohols, and ethoxylated/propoxylated block polymers are also included in this class. The

20 polyoxyethylene surfactants are the most popular members of the nonionic surfactant class.

If the surfactant molecule carries a negative charge when it is dissolved or dispersed in water, the surfactant is classified as anionic. Anionic surfactants include

25 carboxylates such as soaps, acyl lactylates, acyl amides of amino acids, esters of sulfuric acid such as alkyl sulfates and ethoxylated alkyl sulfates, sulfonates such as alkyl benzene sulfonates, acyl isethionates, acyl taurates and sulfosuccinates, and phosphates. The most important

30 members of the anionic surfactant class are the alkyl sulfates and the soaps.

If the surfactant molecule carries a positive charge when it is dissolved or dispersed in water, the surfactant is classified as cationic. Cationic surfactants include

35 quaternary ammonium salts and ethoxylated amines. The quaternary ammonium salts are the most used members of this class.

If the surfactant molecule has the ability to carry either a positive or negative charge, the surfactant is classified as amphoteric. Amphoteric surfactants include acrylic acid derivatives, substituted alkylamides, N-
5   alkylbetaines and phosphatides.

The use of surfactants in drug products, formulations and in emulsions has been reviewed (Rieger, in *Pharmaceutical Dosage Forms*, Marcel Dekker, Inc., New York, NY, **1988**, p. 285).
10

Penetration Enhancers

In one embodiment, the present invention employs various penetration enhancers to effect the efficient delivery of nucleic acids, particularly oligonucleotides,
15   to the skin of animals. Most drugs are present in solution in both ionized and nonionized forms. However, usually only lipid soluble or lipophilic drugs readily cross cell membranes. It has been discovered that even non-lipophilic drugs may cross cell membranes if the membrane to be
20   crossed is treated with a penetration enhancer. In addition to aiding the diffusion of non-lipophilic drugs across cell membranes, penetration enhancers also enhance the permeability of lipophilic drugs.

Penetration enhancers may be classified as belonging
25   to one of five broad categories, *i.e.*, surfactants, fatty acids, bile salts, chelating agents, and non-chelating non-surfactants (Lee *et al.*, *Critical Reviews in Therapeutic Drug Carrier Systems*, **1991**, p.92). Each of the above mentioned classes of penetration enhancers are described
30   below in greater detail.

Surfactants: In connection with the present invention, surfactants (or "surface-active agents") are chemical entities which, when dissolved in an aqueous solution,
35   reduce the surface tension of the solution or the interfacial tension between the aqueous solution and

another liquid, with the result that absorption of
oligonucleotides through the mucosa is enhanced. In
addition to bile salts and fatty acids, these penetration
enhancers include, for example, sodium lauryl sulfate,
polyoxyethylene-9-lauryl ether and polyoxyethylene-20-cetyl
ether) (Lee *et al.*, *Critical Reviews in Therapeutic Drug
Carrier Systems*, **1991**, p.92); and perfluorochemical
emulsions, such as FC-43. Takahashi *et al.*, *J. Pharm.
Pharmacol.*, **1988**, *40*, 252).

Fatty acids: Various fatty acids and their derivatives
which act as penetration enhancers include, for example,
oleic acid, lauric acid, capric acid (n-decanoic acid),
myristic acid, palmitic acid, stearic acid, linoleic acid,
linolenic acid, dicaprate, tricaprate, monoolein (1-
monooleoyl-*rac*-glycerol), dilaurin, caprylic acid,
arachidonic acid, glycerol 1-monocaprate, 1-
dodecylazacycloheptan-2-one, acylcarnitines, acylcholines,
C_{1-10} alkyl esters thereof (*e.g.*, methyl, isopropyl and *t*-
butyl), and mono- and di-glycerides thereof (*i.e.*, oleate,
laurate, caprate, myristate, palmitate, stearate,
linoleate, *etc.*) (Lee *et al.*, *Critical Reviews in
Therapeutic Drug Carrier Systems*, **1991**, p.92; Muranishi,
Critical Reviews in Therapeutic Drug Carrier Systems, **1990**,
7, 1-33; El Hariri *et al.*, J. Pharm. Pharmacol., **1992**, *44*,
651-654).

Bile salts: The physiological role of bile includes
the facilitation of dispersion and absorption of lipids and
fat-soluble vitamins (Brunton, Chapter 38 in: Goodman &
Gilman's *The Pharmacological Basis of Therapeutics*, 9th
Ed., Hardman *et al.* Eds., McGraw-Hill, New York, **1996**, pp.
934-935). Various natural bile salts, and their synthetic
derivatives, act as penetration enhancers. Thus the term

"bile salts" includes any of the naturally occurring
components of bile as well as any of their synthetic
derivatives.   The bile salts of the invention include, for
example, cholic acid (or its pharmaceutically acceptable

5   sodium salt, sodium cholate), dehydrocholic acid (sodium
dehydrocholate), deoxycholic acid (sodium deoxycholate),
glucholic acid (sodium glucholate), glycholic acid (sodium
glycocholate), glycodeoxycholic acid (sodium
glycodeoxycholate), taurocholic acid (sodium taurocholate),

10 taurodeoxycholic acid (sodium taurodeoxycholate),
chenodeoxycholic acid (sodium chenodeoxycholate),
ursodeoxycholic acid (UDCA), sodium tauro-24,25-dihydro-
fusidate (STDHF), sodium glycodihydrofusidate and
polyoxyethylene-9-lauryl ether (POE) (Lee *et al.*, *Critical*

15 *Reviews in Therapeutic Drug Carrier Systems*, **1991**, page 92;
Swinyard, Chapter 39 *In: Remington's Pharmaceutical*
Sciences, 18th Ed., Gennaro, ed., Mack Publishing Co.,
Easton, PA, **1990**, pages 782-783; Muranishi, *Critical*
Reviews in Therapeutic Drug Carrier Systems, **1990**, 7, 1-33;

20 Yamamoto *et al.*, *J. Pharm. Exp. Ther.*, **1992**, *263*, 25;
Yamashita *et al.*, *J. Pharm. Sci.*, **1990**, *79*, 579-583).

     Chelating Agents: Chelating agents, as used in
connection with the present invention, can be defined as

25 compounds that remove metallic ions from solution by
forming complexes therewith, with the result that
absorption of oligonucleotides through the mucosa is
enhanced. With regards to their use as penetration
enhancers in the present invention, chelating agents have

30 the added advantage of also serving as DNase inhibitors, as
most characterized DNA nucleases require a divalent metal
ion for catalysis and are thus inhibited by chelating
agents (Jarrett, *J. Chromatogr.*, **1993**, *618*, 315-339).
Chelating agents of the invention include but are not

35 limited to disodium ethylenediaminetetraacetate (EDTA),

citric acid, salicylates (*e.g.*, sodium salicylate, 5-methoxysalicylate and homovanilate), *N*-acyl derivatives of collagen, laureth-9 and *N*-amino acyl derivatives of beta-diketones (enamines)(Lee *et al.*, *Critical Reviews in*

5 *Therapeutic Drug Carrier Systems*, **1991**, page 92; Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, **1990**, 7, 1-33; Buur *et al.*, *J. Control Rel.*, **1990**, *14*, 43-51).

Non-chelating non-surfactants: As used herein, non-
10 chelating non-surfactant penetration enhancing compounds can be defined as compounds that demonstrate insignificant activity as chelating agents or as surfactants but that nonetheless enhance absorption of oligonucleotides through the alimentary mucosa (Muranishi, *Critical Reviews in*

15 *Therapeutic Drug Carrier Systems*, **1990**, 7, 1-33). This class of penetration enhancers include, for example, unsaturated cyclic ureas, 1-alkyl- and 1-alkenylazacyclo-alkanone derivatives (Lee *et al.*, *Critical Reviews in Therapeutic Drug Carrier Systems*, **1991**, page 92); and non-

20 steroidal anti-inflammatory agents such as diclofenac sodium, indomethacin and phenylbutazone (Yamashita *et al.*, *J. Pharm. Pharmacol.*, **1987**, *39*, 621-626).

Agents that enhance uptake of oligonucleotides at the cellular level may also be added to the pharmaceutical and

25 other compositions of the present invention. For example, cationic lipids, such as lipofectin (Junichi *et al*, U.S. Patent No. 5,705,188), cationic glycerol derivatives, and polycationic molecules, such as polylysine (Lollo *et al.*, PCT Application WO 97/30731), are also known to enhance the

30 cellular uptake of oligonucleotides.

Other agents may be utilized to enhance the penetration of the administered nucleic acids, including glycols such as ethylene glycol and propylene glycol,

pyrrols such as 2-pyrrol, azones, and terpenes such as limonene and menthone.

Carriers

Certain compositions of the present invention also incorporate carrier compounds in the formulation. As used herein, "carrier compound" or "carrier" can refer to a nucleic acid, or analog thereof, which is inert (*i.e.*, does not possess biological activity *per se*) but is recognized as a nucleic acid by *in vivo* processes that reduce the bioavailability of a nucleic acid having biological activity by, for example, degrading the biologically active nucleic acid or promoting its removal from circulation. The coadministration of a nucleic acid and a carrier compound, typically with an excess of the latter substance, can result in a substantial reduction of the amount of nucleic acid recovered in the liver, kidney or other extracirculatory reservoirs, presumably due to competition between the carrier compound and the nucleic acid for a common receptor. For example, the recovery of a partially phosphorothioate oligonucleotide in hepatic tissue can be reduced when it is coadministered with polyinosinic acid, dextran sulfate, polycytidic acid or 4-acetamido-4'isothiocyano-stilbene-2,2'-disulfonic acid (Miyao *et al.*, *Antisense Res. Dev.*, **1995**, *5*, 115-121; Takakura *et al.*, *Antisense & Nucl. Acid Drug Dev.*, **1996**, *6*, 177-183).

Excipients

In contrast to a carrier compound, a "pharmaceutical carrier" or "excipient" is a pharmaceutically acceptable solvent, suspending agent or any other pharmacologically inert vehicle for delivering one or more nucleic acids to an animal. The excipient may be liquid or solid and is selected, with the planned manner of administration in mind, so as to provide for the desired bulk, consistency, *etc.*, when combined with a nucleic acid and the other

components of a given pharmaceutical composition. Typical pharmaceutical carriers include, but are not limited to, binding agents (*e.g.*, pregelatinized maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose,

5   *etc.*); fillers (*e.g.*, lactose and other sugars, microcrystalline cellulose, pectin, gelatin, calcium sulfate, ethyl cellulose, polyacrylates or calcium hydrogen phosphate, *etc.*); lubricants (*e.g.*, magnesium stearate, talc, silica, colloidal silicon dioxide, stearic acid,

10 metallic stearates, hydrogenated vegetable oils, corn starch, polyethylene glycols, sodium benzoate, sodium acetate, *etc.*); disintegrants (*e.g.*, starch, sodium starch glycolate, *etc.*); and wetting agents (*e.g.*, sodium lauryl sulphate, *etc.*).

15   Pharmaceutically acceptable organic or inorganic excipient suitable for non-parenteral administration which do not deleteriously react with nucleic acids can also be used to formulate the compositions of the present invention. Suitable pharmaceutically acceptable carriers

20 include, but are not limited to, water, salt solutions, alcohols, polyethylene glycols, gelatin, lactose, amylose, magnesium stearate, talc, silicic acid, viscous paraffin, hydroxymethylcellulose, polyvinylpyrrolidone and the like.

        Formulations for topical administration of nucleic

25 acids may include sterile and non-sterile aqueous solutions, non-aqueous solutions in common solvents such as alcohols, or solutions of the nucleic acids in liquid or solid oil bases. The solutions may also contain buffers, diluents and other suitable additives. Pharmaceutically

30 acceptable organic or inorganic excipients suitable for non-parenteral administration which do not deleteriously react with nucleic acids can be used.

        Suitable pharmaceutically acceptable excipients include, but are not limited to, water, salt solutions,

35 alcohol, polyethylene glycols, gelatin, lactose, amylose,

magnesium stearate, talc, silicic acid, viscous paraffin, hydroxymethylcellulose, polyvinylpyrrolidone and the like.

Other Components

5   The compositions of the present invention may additionally contain other adjunct components conventionally found in pharmaceutical compositions, at their art-established usage levels. Thus, for example, the compositions may contain additional, compatible,
10   pharmaceutically-active materials such as, for example, antipruritics, astringents, local anesthetics or anti-inflammatory agents, or may contain additional materials useful in physically formulating various dosage forms of the compositions of the present invention, such as
15   dyes, flavoring agents, preservatives, antioxidants, opacifiers, thickening agents and stabilizers. However, such materials, when added, should not unduly interfere with the biological activities of the components of the compositions of the present invention. The formulations
20   can be sterilized and, if desired, mixed with auxiliary agents, *e.g.*, lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers, colorings, flavorings and/or aromatic substances and the like which do not deleteriously interact
25   with the nucleic acid(s) of the formulation.

       Aqueous suspensions may contain substances which increase the viscosity of the suspension including, for example, sodium carboxymethylcellulose, sorbitol and/or dextran. The suspension may also contain stabilizers.
30   Certain embodiments of the invention provide pharmaceutical compositions containing (a) one or more antisense compounds and (b) one or more other chemotherapeutic agents which function by a non-antisense mechanism. Examples of such chemotherapeutic agents include
35   but are not limited to daunorubicin, daunomycin, dactinomycin, doxorubicin, epirubicin, idarubicin, esorubicin, bleomycin, mafosfamide, ifosfamide, cytosine

arabinoside, bis-chloroethylnitrosurea, busulfan, mitomycin C, actinomycin D, mithramycin, prednisone, hydroxyprogesterone, testosterone, tamoxifen, dacarbazine, procarbazine, hexamethylmelamine, pentamethylmelamine,

5   mitoxantrone, amsacrine, chlorambucil, methylcyclohexylnitrosurea, nitrogen mustards, melphalan, cyclophosphamide, 6-mercaptopurine, 6-thioguanine, cytarabine, 5-azacytidine, hydroxyurea, deoxycoformycin, 4-hydroxyperoxycyclophosphoramide, 5-fluorouracil (5-FU), 5-

10   fluorodeoxyuridine (5-FUdR), methotrexate (MTX), colchicine, taxol, vincristine, vinblastine, etoposide (VP-16), trimetrexate, irinotecan, topotecan, gemcitabine, teniposide, cisplatin and diethylstilbestrol (DES). See, generally, *The Merck Manual of Diagnosis and Therapy*, 15th

15   Ed. **1987**, pp. 1206-1228, Berkow *et al.*, eds., Rahway, N.J. When used with the compounds of the invention, such chemotherapeutic agents may be used individually (*e.g.*, 5-FU and oligonucleotide), sequentially (*e.g.*, 5-FU and oligonucleotide for a period of time followed by MTX and

20   oligonucleotide), or in combination with one or more other such chemotherapeutic agents (*e.g.*, 5-FU, MTX and oligonucleotide, or 5-FU, radiotherapy and oligonucleotide). Anti-inflammatory drugs, including but not limited to nonsteroidal anti-inflammatory drugs and

25   corticosteroids, and antiviral drugs, including but not limited to ribivirin, vidarabine, acyclovir and ganciclovir, may also be combined in compositions of the invention. See, generally, *The Merck Manual of Diagnosis and Therapy*, 15th Ed., Berkow et al., eds., **1987**, Rahway,

30   N.J., pages 2499-2506 and 46-49, respectively). Other non-antisense chemotherapeutic agents are also within the scope of this invention. Two or more combined compounds may be used together or sequentially.

     In another related embodiment, compositions of the
35   invention may contain one or more antisense compounds, particularly oligonucleotides, targeted to a first nucleic

acid and one or more additional antisense compounds targeted to a second nucleic acid target. Numerous examples of antisense compounds are known in the art. Two or more combined compounds may be used together or sequentially.

5   The formulation of therapeutic compositions and their subsequent administration is believed to be within the skill of those in the art. Dosing is dependent on severity and responsiveness of the disease state to be treated, with the course of treatment lasting from several days to

10   several months, or until a cure is effected or a diminution of the disease state is achieved. Optimal dosing schedules can be calculated from measurements of drug accumulation in the body of the patient. Persons of ordinary skill can easily determine optimum dosages, dosing methodologies and

15   repetition rates. Optimum dosages may vary depending on the relative potency of individual oligonucleotides, and can generally be estimated based on EC_{50}s found to be effective in in vitro and in vivo animal models. In general, dosage is from 0.01 ug to 100 g per kg of body

20   weight, and may be given once or more daily, weekly, monthly or yearly, or even once every 2 to 20 years. Persons of ordinary skill in the art can easily estimate repetition rates for dosing based on measured residence times and concentrations of the drug in bodily fluids or

25   tissues. Following successful treatment, it may be desirable to have the patient undergo maintenance therapy to prevent the recurrence of the disease state, wherein the oligonucleotide is administered in maintenance doses, ranging from 0.01 ug to 100 g per kg of body weight, once

30   or more daily, to once every 20 years.

      While the present invention has been described with specificity in accordance with certain of its preferred embodiments, the following examples serve only to illustrate the invention and are not intended to limit the

35   same.

EXAMPLES

Example 1

Nucleoside Phosphoramidites for Oligonucleotide Synthesis

5 **Deoxy and 2'-alkoxy amidites**

2'-Deoxy and 2'-methoxy beta-cyanoethyldiisopropyl phosphoramidites were purchased from commercial sources (e.g. Chemgenes, Needham MA or Glen Research, Inc. Sterling VA). Other 2'-O-alkoxy substituted nucleoside amidites are

10 prepared as described in U.S. Patent 5,506,351, herein incorporated by reference. For oligonucleotides synthesized using 2'-alkoxy amidites, the standard cycle for unmodified oligonucleotides was utilized, except the wait step after pulse delivery of tetrazole and base was

15 increased to 360 seconds.

Oligonucleotides containing 5-methyl-2'-deoxycytidine (5-Me-C) nucleotides were synthesized according to published methods [Sanghvi, et. al., *Nucleic Acids Research*, **1993**, *21*, 3197-3203] using commercially available

20 phosphoramidites (Glen Research, Sterling VA or ChemGenes, Needham MA).

2'-Fluoro amidites

2'-Fluorodeoxyadenosine amidites

25 2'-fluoro oligonucleotides were synthesized as described previously [Kawasaki, et. al., *J. Med. Chem.*, **1993**, *36*, 831-841] and United States patent 5,670,633, herein incorporated by reference. Briefly, the protected nucleoside N6-benzoyl-2'-deoxy-2'-fluoroadenosine was

30 synthesized utilizing commercially available 9-beta-D-arabinofuranosyladenine as starting material and by modifying literature procedures whereby the 2'-alpha-fluoro atom is introduced by a S_N2-displacement of a 2'-beta-trityl group. Thus N6-benzoyl-9-beta-D-arabinofuranosyladenine

35 was selectively protected in moderate yield as the 3',5'-

ditetrahydropyranyl (THP) intermediate. Deprotection of
the THP and N6-benzoyl groups was accomplished using
standard methodologies and standard methods were used to
obtain the 5'-dimethoxytrityl-(DMT) and 5'-DMT-3'-
5   phosphoramidite intermediates.

2'-Fluorodeoxyguanosine

The synthesis of 2'-deoxy-2'-fluoroguanosine was
accomplished using tetraisopropyldisiloxanyl (TPDS)
10   protected 9-beta-D-arabinofuranosylguanine as starting
material, and conversion to the intermediate diisobutyryl-
arabinofuranosylguanosine. Deprotection of the TPDS group
was followed by protection of the hydroxyl group with THP
to give diisobutyryl di-THP protected
15   arabinofuranosylguanine. Selective O-deacylation and
triflation was followed by treatment of the crude product
with fluoride, then deprotection of the THP groups.
Standard methodologies were used to obtain the 5'-DMT- and
5'-DMT-3'-phosphoramidites.
20

2'-Fluorouridine

Synthesis of 2'-deoxy-2'-fluorouridine was
accomplished by the modification of a literature procedure
in which 2,2'-anhydro-1-beta-D-arabinofuranosyluracil was
25   treated with 70% hydrogen fluoride-pyridine. Standard
procedures were used to obtain the 5'-DMT and 5'-DMT-
3'phosphoramidites.

2'-Fluorodeoxycytidine

30   2'-deoxy-2'-fluorocytidine was synthesized via
amination of 2'-deoxy-2'-fluorouridine, followed by
selective protection to give N4-benzoyl-2'-deoxy-2'-
fluorocytidine. Standard procedures were used to obtain
the 5'-DMT and 5'-DMT-3'phosphoramidites.
35

2'-O-(2-Methoxyethyl) modified amidites

2'-O-Methoxyethyl-substituted nucleoside amidites are prepared as follows, or alternatively, as per the methods of Martin, P., *Helvetica Chimica Acta*, **1995**, *78*, 486-504.

2,2'-Anhydro[1-(beta-D-arabinofuranosyl)-5-methyluridine]

5-Methyluridine (ribosylthymine, commercially available through Yamasa, Choshi, Japan) (72.0 g, 0.279 M), diphenylcarbonate (90.0 g, 0.420 M) and sodium bicarbonate (2.0 g, 0.024 M) were added to DMF (300 mL). The mixture was heated to reflux, with stirring, allowing the evolved carbon dioxide gas to be released in a controlled manner. After 1 hour, the slightly darkened solution was concentrated under reduced pressure. The resulting syrup was poured into diethylether (2.5 L), with stirring. The product formed a gum. The ether was decanted and the residue was dissolved in a minimum amount of methanol (ca. 400 mL). The solution was poured into fresh ether (2.5 L) to yield a stiff gum. The ether was decanted and the gum was dried in a vacuum oven (60°C at 1 mm Hg for 24 h) to give a solid that was crushed to a light tan powder (57 g, 85% crude yield). The NMR spectrum was consistent with the structure, contaminated with phenol as its sodium salt (ca. 5%). The material was used as is for further reactions (or it can be purified further by column chromatography using a gradient of methanol in ethyl acetate (10-25%) to give a white solid, mp 222-4°C).

2'-O-Methoxyethyl-5-methyluridine

2,2'-Anhydro-5-methyluridine (195 g, 0.81 M), tris(2-methoxyethyl)borate (231 g, 0.98 M) and 2-methoxyethanol (1.2 L) were added to a 2 L stainless steel pressure vessel and placed in a pre-heated oil bath at 160°C. After heating for 48 hours at 155-160°C, the vessel was opened and the

solution evaporated to dryness and triturated with MeOH
(200 mL). The residue was suspended in hot acetone (1 L).
The insoluble salts were filtered, washed with acetone (150
mL) and the filtrate evaporated. The residue (280 g) was
5   dissolved in CH_3CN (600 mL) and evaporated. A silica gel
column (3 kg) was packed in CH_2Cl_2/acetone/MeOH (20:5:3)
containing 0.5% Et_3NH. The residue was dissolved in CH_2Cl_2
(250 mL) and adsorbed onto silica (150 g) prior to loading
onto the column. The product was eluted with the packing
10   solvent to give 160 g (63%) of product. Additional
material was obtained by reworking impure fractions.

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine

2'-O-Methoxyethyl-5-methyluridine (160 g, 0.506 M) was
15   co-evaporated with pyridine (250 mL) and the dried residue
dissolved in pyridine (1.3 L). A first aliquot of
dimethoxytrityl chloride (94.3 g, 0.278 M) was added and
the mixture stirred at room temperature for one hour. A
second aliquot of dimethoxytrityl chloride (94.3 g, 0.278
20   M) was added and the reaction stirred for an additional one
hour. Methanol (170 mL) was then added to stop the
reaction. HPLC showed the presence of approximately 70%
product. The solvent was evaporated and triturated with
CH_3CN (200 mL). The residue was dissolved in $CHCl_3$ (1.5 L)
25   and extracted with 2x500 mL of saturated $NaHCO_3$ and 2x500 mL
of saturated NaCl. The organic phase was dried over Na_2SO_4,
filtered and evaporated. 275 g of residue was obtained.
The residue was purified on a 3.5 kg silica gel column,
packed and eluted with EtOAc/hexane/acetone (5:5:1)
30   containing 0.5% Et_3NH. The pure fractions were evaporated
to give 164 g of product. Approximately 20 g additional
was obtained from the impure fractions to give a total
yield of 183 g (57%).

3'-O-Acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine (106 g, 0.167 M), DMF/pyridine (750 mL of a 3:1 mixture
5   prepared from 562 mL of DMF and 188 mL of pyridine) and acetic anhydride (24.38 mL, 0.258 M) were combined and stirred at room temperature for 24 hours. The reaction was monitored by TLC by first quenching the TLC sample with the addition of MeOH. Upon completion of the reaction, as
10 judged by TLC, MeOH (50 mL) was added and the mixture evaporated at 35°C. The residue was dissolved in $CHCl_3$ (800 mL) and extracted with 2x200 mL of saturated sodium bicarbonate and 2x200 mL of saturated NaCl. The water layers were back extracted with 200 mL of $CHCl_3$. The
15 combined organics were dried with sodium sulfate and evaporated to give 122 g of residue (approx. 90% product). The residue was purified on a 3.5 kg silica gel column and eluted using EtOAc/hexane(4:1). Pure product fractions were evaporated to yield 96 g (84%). An additional 1.5 g
20 was recovered from later fractions.

3'-O-Acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-4-triazoleuridine

A first solution was prepared by dissolving 3'-O-
25 acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine (96 g, 0.144 M) in CH_3CN (700 mL) and set aside. Triethylamine (189 mL, 1.44 M) was added to a solution of triazole (90 g, 1.3 M) in CH_3CN (1 L), cooled to -5°C and stirred for 0.5 h using an overhead stirrer. $POCl_3$
30 was added dropwise, over a 30 minute period, to the stirred solution maintained at 0-10°C, and the resulting mixture stirred for an additional 2 hours. The first solution was added dropwise, over a 45 minute period, to the latter solution. The resulting reaction mixture was stored
35 overnight in a cold room. Salts were filtered from the

reaction mixture and the solution was evaporated. The residue was dissolved in EtOAc (1 L) and the insoluble solids were removed by filtration. The filtrate was washed with 1x300 mL of NaHCO$_3$ and 2x300 mL of saturated NaCl,

5   dried over sodium sulfate and evaporated. The residue was triturated with EtOAc to give the title compound.

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine

10   A solution of 3'-O-acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-4-triazoleuridine (103 g, 0.141 M) in dioxane (500 mL) and NH$_4$OH (30 mL) was stirred at room temperature for 2 hours. The dioxane solution was evaporated and the residue azeotroped with MeOH (2x200 mL).

15   The residue was dissolved in MeOH (300 mL) and transferred to a 2 liter stainless steel pressure vessel. MeOH (400 mL) saturated with NH$_3$ gas was added and the vessel heated to 100°C for 2 hours (TLC showed complete conversion). The vessel contents were evaporated to dryness and the residue

20   was dissolved in EtOAc (500 mL) and washed once with saturated NaCl (200 mL). The organics were dried over sodium sulfate and the solvent was evaporated to give 85 g (95%) of the title compound.

25   ### N4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyl-cytidine (85 g, 0.134 M) was dissolved in DMF (800 mL) and benzoic anhydride (37.2 g, 0.165 M) was added with

30   stirring. After stirring for 3 hours, TLC showed the reaction to be approximately 95% complete. The solvent was evaporated and the residue azeotroped with MeOH (200 mL). The residue was dissolved in CHCl$_3$ (700 mL) and extracted with saturated NaHCO$_3$ (2x300 mL) and saturated NaCl (2x300

35   mL), dried over MgSO$_4$ and evaporated to give a residue (96 g). The residue was chromatographed on a 1.5 kg silica

column using EtOAc/hexane (1:1) containing 0.5% Et$_3$NH as the
eluting solvent. The pure product fractions were
evaporated to give 90 g (90%) of the title compound.

**N4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-
methylcytidine-3'-amidite**

N4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-
methylcytidine (74 g, 0.10 M) was dissolved in CH$_2$Cl$_2$ (1 L).
Tetrazole diisopropylamine (7.1 g) and 2-cyanoethoxy-tetra-
(isopropyl)phosphite (40.5 mL, 0.123 M) were added with
stirring, under a nitrogen atmosphere. The resulting
mixture was stirred for 20 hours at room temperature (TLC
showed the reaction to be 95% complete). The reaction
mixture was extracted with saturated NaHCO$_3$ (1x300 mL) and
saturated NaCl (3x300 mL). The aqueous washes were back-
extracted with CH$_2$Cl$_2$ (300 mL), and the extracts were
combined, dried over MgSO$_4$ and concentrated. The residue
obtained was chromatographed on a 1.5 kg silica column
using EtOAc/hexane (3:1) as the eluting solvent. The pure
fractions were combined to give 90.6 g (87%) of the title
compound.

**2'-O-(Aminooxyethyl) nucleoside amidites and 2'-O-
(dimethylaminooxyethyl) nucleoside amidites**

2'-(Dimethylaminooxyethoxy) nucleoside amidites

2'-(Dimethylaminooxyethoxy) nucleoside amidites [also
known in the art as 2'-O-(dimethylaminooxyethyl) nucleoside
amidites] are prepared as described in the following
paragraphs. Adenosine, cytidine and guanosine nucleoside
amidites are prepared similarly to the thymidine (5-
methyluridine) except the exocyclic amines are protected
with a benzoyl moiety in the case of adenosine and cytidine
and with isobutyryl in the case of guanosine.

5'-O-tert-Butyldiphenylsilyl-O²-2'-anhydro-5-methyluridine

O²-2'-anhydro-5-methyluridine (Pro. Bio. Sint., Varese, Italy, 100.0g, 0.416 mmol), dimethylaminopyridine (0.66g, 0.013eq, 0.0054mmol) were dissolved in dry pyridine (500 ml) at ambient temperature under an argon atmosphere and with mechanical stirring. *tert*-Butyldiphenylchlorosilane (125.8g, 119.0mL, 1.1eq, 0.458mmol) was added in one portion. The reaction was stirred for 16 h at ambient temperature. TLC (Rf 0.22, ethyl acetate) indicated a complete reaction. The solution was concentrated under reduced pressure to a thick oil. This was partitioned between dichloromethane (1 L) and saturated sodium bicarbonate (2x1 L) and brine (1 L). The organic layer was dried over sodium sulfate and concentrated under reduced pressure to a thick oil. The oil was dissolved in a 1:1 mixture of ethyl acetate and ethyl ether (600mL) and the solution was cooled to -10°C. The resulting crystalline product was collected by filtration, washed with ethyl ether (3x200 mL) and dried (40°C, 1mm Hg, 24 h) to 149g (74.8%) of white solid. TLC and NMR were consistent with pure product.

5'-O-tert-Butyldiphenylsilyl-2'-O-(2-hydroxyethyl)-5-methyluridine

In a 2 L stainless steel, unstirred pressure reactor was added borane in tetrahydrofuran (1.0 M, 2.0 eq, 622 mL). In the fume hood and with manual stirring, ethylene glycol (350 mL, excess) was added cautiously at first until the evolution of hydrogen gas subsided. 5'-O-tert-Butyldiphenylsilyl-O²-2'-anhydro-5-methyluridine (149 g, 0.311 mol) and sodium bicarbonate (0.074 g, 0.003 eq) were added with manual stirring. The reactor was sealed and heated in an oil bath until an internal temperature of 160 °C was reached and then maintained for 16 h (pressure < 100 psig). The reaction vessel was cooled to ambient and

opened. TLC (Rf 0.67 for desired product and Rf 0.82 for
ara-T side product, ethyl acetate) indicated about 70%
conversion to the product. In order to avoid additional
side product formation, the reaction was stopped,

5   concentrated under reduced pressure (10 to 1mm Hg) in a
warm water bath (40-100°C) with the more extreme conditions
used to remove the ethylene glycol. [Alternatively, once
the low boiling solvent is gone, the remaining solution can
be partitioned between ethyl acetate and water. The

10   product will be in the organic phase.] The residue was
purified by column chromatography (2kg silica gel, ethyl
acetate-hexanes gradient 1:1 to 4:1). The appropriate
fractions were combined, stripped and dried to product as a
white crisp foam (84g, 50%), contaminated starting material

15   (17.4g) and pure reusable starting material 20g. The yield
based on starting material less pure recovered starting
material was 58%. TLC and NMR were consistent with 99%
pure product.

20   **2'-O-([2-phthalimidoxy)ethyl]-5'-*t*-butyldiphenylsilyl-**
          5-methyluridine

          5'-O-tert-Butyldiphenylsilyl-2'-O-(2-hydroxyethyl)-5-
methyluridine (20g, 36.98mmol) was mixed with
triphenylphosphine (11.63g, 44.36mmol) and N-

25   hydroxyphthalimide (7.24g, 44.36mmol). It was then dried
over P_2O_5 under high vacuum for two days at 40°C. The
reaction mixture was flushed with argon and dry THF
(369.8mL, Aldrich, sure seal bottle) was added to get a
clear solution. Diethyl-azodicarboxylate (6.98mL,

30   44.36mmol) was added dropwise to the reaction mixture. The
rate of addition is maintained such that resulting deep red
coloration is just discharged before adding the next drop.
After the addition was complete, the reaction was stirred
for 4 hrs. By that time TLC showed the completion of the

35   reaction (ethylacetate:hexane, 60:40). The solvent was
evaporated in vacuum. Residue obtained was placed on a

flash column and eluted with ethyl acetate:hexane (60:40), to get 2'-O-([2-phthalimidoxy)ethyl]-5'-*t*-butyldiphenylsilyl-5-methyluridine as white foam (21.819 g, 86%).

5'-O-*tert*-butyldiphenylsilyl-2'-O-[(2-formadoximinooxy)ethyl]-5-methyluridine

2'-O-([2-phthalimidoxy)ethyl]-5'-*t*-butyldiphenylsilyl-5-methyluridine (3.1g, 4.5mmol) was dissolved in dry CH_2Cl_2 (4.5mL) and methylhydrazine (300mL, 4.64mmol) was added dropwise at -10°C to 0°C. After 1 h the mixture was filtered, the filtrate was washed with ice cold CH_2Cl_2 and the combined organic phase was washed with water, brine and dried over anhydrous Na_2SO_4. The solution was concentrated to get 2'-O-(aminooxyethyl) thymidine, which was then dissolved in MeOH (67.5mL). To this formaldehyde (20% aqueous solution, w/w, 1.1 eq.) was added and the resulting mixture was strirred for 1 h. Solvent was removed under vacuum; residue chromatographed to get 5'-O-*tert*-butyldiphenylsilyl-2'-O-[(2-formadoximinooxy) ethyl]-5-methyluridine as white foam (1.95 g, 78%).

5'-O-*tert*-Butyldiphenylsilyl-2'-O-[N,N-dimethylaminooxyethyl]-5-methyluridine

5'-O-*tert*-butyldiphenylsilyl-2'-O-[(2-formadoximinooxy)ethyl]-5-methyluridine (1.77g, 3.12mmol) was dissolved in a solution of 1M pyridinium p-toluenesulfonate (PPTS) in dry MeOH (30.6mL). Sodium cyanoborohydride (0.39g, 6.13mmol) was added to this solution at 10°C under inert atmosphere. The reaction mixture was stirred for 10 minutes at 10°C. After that the reaction vessel was removed from the ice bath and stirred at room temperature for 2 h, the reaction monitored by TLC (5% MeOH in CH_2Cl_2). Aqueous $NaHCO_3$ solution (5%, 10mL) was

added and extracted with ethyl acetate (2x20mL). Ethyl acetate phase was dried over anhydrous Na$_2$SO$_4$, evaporated to dryness. Residue was dissolved in a solution of 1M PPTS in MeOH (30.6mL). Formaldehyde (20% w/w, 30mL, 3.37mmol) was

5   added and the reaction mixture was stirred at room temperature for 10 minutes. Reaction mixture cooled to 10°C in an ice bath, sodium cyanoborohydride (0.39g, 6.13mmol) was added and reaction mixture stirred at 10°C for 10 minutes. After 10 minutes, the reaction mixture was

10   removed from the ice bath and stirred at room temperature for 2 hrs. To the reaction mixture 5% NaHCO$_3$ (25mL) solution was added and extracted with ethyl acetate (2x25mL). Ethyl acetate layer was dried over anhydrous Na$_2$SO$_4$ and evaporated to dryness. The residue obtained was

15   purified by flash column chromatography and eluted with 5% MeOH in CH$_2$Cl$_2$ to get 5'-O-*tert*-butyldiphenylsilyl-2'-O-[N,N-dimethylaminooxyethyl]-5-methyluridine as a white foam (14.6g, 80%).

20   **2'-O-(dimethylaminooxyethyl)-5-methyluridine**
         Triethylamine trihydrofluoride (3.91mL, 24.0mmol) was dissolved in dry THF and triethylamine (1.67mL, 12mmol, dry, kept over KOH). This mixture of triethylamine-2HF was then added to 5'-O-*tert*-butyldiphenylsilyl-2'-O-[N,N-

25   dimethylaminooxyethyl]-5-methyluridine (1.40g, 2.4mmol) and stirred at room temperature for 24 hrs. Reaction was monitored by TLC (5% MeOH in CH$_2$Cl$_2$). Solvent was removed under vacuum and the residue placed on a flash column and eluted with 10% MeOH in CH$_2$Cl$_2$ to get 2'-O-

30   (dimethylaminooxyethyl)-5-methyluridine (766mg, 92.5%).

       5'-O-DMT-2'-O-(dimethylaminooxyethyl)-5-methyluridine
       2'-O-(dimethylaminooxyethyl)-5-methyluridine (750mg, 2.17mmol) was dried over P$_2$O$_5$ under high vacuum overnight at

35   40°C. It was then co-evaporated with anhydrous pyridine

(20mL). The residue obtained was dissolved in pyridine
(11mL) under argon atmosphere. 4-dimethylaminopyridine
(26.5mg, 2.60mmol), 4,4'-dimethoxytrityl chloride (880mg,
2.60mmol) was added to the mixture and the reaction mixture
5   was stirred at room temperature until all of the starting
material disappeared. Pyridine was removed under vacuum
and the residue chromatographed and eluted with 10% MeOH in
CH_2Cl_2 (containing a few drops of pyridine) to get 5'-O-DMT-
2'-O-(dimethylamino-oxyethyl)-5-methyluridine (1.13g, 80%).
10

5'-O-DMT-2'-O-(2-N,N-dimethylaminooxyethyl)-5-

methyluridine-3'-[(2-cyanoethyl)-N,N-

diisopropylphosphoramidite]

5'-O-DMT-2'-O-(dimethylaminooxyethyl)-5-methyluridine
15 (1.08g, 1.67mmol) was co-evaporated with toluene (20mL).
To the residue N,N-diisopropylamine tetrazonide (0.29g,
1.67mmol) was added and dried over P_2O_5 under high vacuum
overnight at 40°C. Then the reaction mixture was dissolved
in anhydrous acetonitrile (8.4mL) and 2-cyanoethyl-
20 N,N,N^1,N^1-tetraisopropylphosphoramidite (2.12mL, 6.08mmol)
was added. The reaction mixture was stirred at ambient
temperature for 4 hrs under inert atmosphere. The progress
of the reaction was monitored by TLC (hexane:ethyl acetate
1:1). The solvent was evaporated, then the residue was
25 dissolved in ethyl acetate (70mL) and washed with 5%
aqueous $NaHCO_3$ (40mL). Ethyl acetate layer was dried over
anhydrous Na_2SO_4 and concentrated. Residue obtained was
chromatographed (ethyl acetate as eluent) to get 5'-O-DMT-
2'-O-(2-N,N-dimethylaminooxyethyl)-5-methyluridine-3'-[(2-
30 cyanoethyl)-N,N-diisopropylphosphoramidite] as a foam
(1.04g, 74.9%).

2'-(Aminooxyethoxy) nucleoside amidites

2'-(Aminooxyethoxy) nucleoside amidites [also known in
35 the art as 2'-O-(aminooxyethyl) nucleoside amidites] are
prepared as described in the following paragraphs.

Adenosine, cytidine and thymidine nucleoside amidites are
prepared similarly.

N2-isobutyryl-6-O-diphenylcarbamoyl-2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite]

The 2'-O-aminooxyethyl guanosine analog may be
obtained by selective 2'-O-alkylation of diaminopurine
riboside. Multigram quantities of diaminopurine riboside
may be purchased from Schering AG (Berlin) to provide 2'-O-
(2-ethylacetyl) diaminopurine riboside along with a minor
amount of the 3'-O-isomer. 2'-O-(2-ethylacetyl)
diaminopurine riboside may be resolved and converted to 2'-
O-(2-ethylacetyl)guanosine by treatment with adenosine
deaminase. (McGee, D. P. C., Cook, P. D., Guinosso, C. J.,
WO 94/02501 A1 940203.) Standard protection procedures
should afford 2'-O-(2-ethylacetyl)-5'-O-(4,4'-
dimethoxytrityl)guanosine and 2-N-isobutyryl-6-O-
diphenylcarbamoyl-2'-O-(2-ethylacetyl)-5'-O-(4,4'-
dimethoxytrityl)guanosine which may be reduced to provide
2-N-isobutyryl-6-O-diphenylcarbamoyl-2'-O-(2-hydroxyethyl)-
5'-O-(4,4'-dimethoxytrityl)guanosine. As before the
hydroxyl group may be displaced by N-hydroxyphthalimide via
a Mitsunobu reaction, and the protected nucleoside may
phosphitylated as usual to yield 2-N-isobutyryl-6-O-
diphenylcarbamoyl-2'-O-([2-phthalmidoxy]ethyl)-5'-O-(4,4'-
dimethoxytrityl)guanosine-3'-[(2-cyanoethyl)-N,N-
diisopropylphosphoramidite].

2'-dimethylaminoethoxyethoxy (2'-DMAEOE) nucleoside amidites

2'-dimethylaminoethoxyethoxy nucleoside amidites (also
known in the art as 2'-O-dimethylaminoethoxyethyl, i.e.,
2'-O-CH$_2$-O-CH$_2$-N(CH$_2$)$_2$, or 2'-DMAEOE nucleoside amidites) are
prepared as follows. Other nucleoside amidites are prepared
similarly.

2'-O-[2(2-N,N-dimethylaminoethoxy)ethyl]-5-methyl uridine

2[2-(Dimethylamino)ethoxy]ethanol (Aldrich, 6.66 g, 50 mmol) is slowly added to a solution of borane in tetra-hydrofuran (1 M, 10 mL, 10 mmol) with stirring in a 100 mL bomb. Hydrogen gas evolves as the solid dissolves. $O^2-,2'-$ anhydro-5-methyluridine (1.2 g, 5 mmol), and sodium bicarbonate (2.5 mg) are added and the bomb is sealed, placed in an oil bath and heated to 155°C for 26 hours. The bomb is cooled to room temperature and opened. The crude solution is concentrated and the residue partitioned between water (200 mL) and hexanes (200 mL). The excess phenol is extracted into the hexane layer. The aqueous layer is extracted with ethyl acetate (3x200 mL) and the combined organic layers are washed once with water, dried over anhydrous sodium sulfate and concentrated. The residue is columned on silica gel using methanol/methylene chloride 1:20 (which has 2% triethylamine) as the eluent. As the column fractions are concentrated a colorless solid forms which is collected to give the title compound as a white solid.

5'-O-dimethoxytrityl-2'-O-[2(2-N,N-dimethylaminoethoxy)-ethyl)]-5-methyl uridine

To 0.5 g (1.3 mmol) of 2'-O-[2(2-N,N-dimethylamino-ethoxy)ethyl)]-5-methyl uridine in anhydrous pyridine (8 mL), triethylamine (0.36 mL) and dimethoxytrityl chloride (DMT-Cl, 0.87 g, 2 eq.) are added and stirred for 1 hour. The reaction mixture is poured into water (200 mL) and extracted with CH_2Cl_2 (2x200 mL). The combined CH_2Cl_2 layers are washed with saturated $NaHCO_3$ solution, followed by saturated NaCl solution and dried over anhydrous sodium sulfate. Evaporation of the solvent followed by silica gel chromatography using $MeOH:CH_2Cl_2:Et_3N$ (20:1, v/v, with 1% triethylamine) gives the title compound.

5'-O-Dimethoxytrityl-2'-O-[2(2-N,N-

dimethylaminoethoxy)ethyl)]-5-methyl uridine-3'-O-

(cyanoethyl-N,N-diisopropyl)phosphoramidite

Diisopropylaminotetrazolide (0.6 g) and 2-cyanoethoxy-
5 N,N-diisopropyl phosphoramidite (1.1 mL, 2 eq.) are added
to a solution of 5'-O-dimethoxytrityl-2'-O-[2(2-N,N-
dimethylaminoethoxy)ethyl)]-5-methyluridine (2.17 g, 3
mmol) dissolved in CH$_2$Cl$_2$ (20 mL) under an atmosphere of
argon. The reaction mixture is stirred overnight and the
10 solvent evaporated. The resulting residue is purified by
silica gel flash column chromatography with ethyl acetate
as the eluent to give the title compound.

Example 2

15 **Oligonucleotide synthesis**

Unsubstituted and substituted phosphodiester (P=O)
oligonucleotides are synthesized on an automated DNA
synthesizer (Applied Biosystems model 380B) using standard
phosphoramidite chemistry with oxidation by iodine.
20 Phosphorothioates (P=S) are synthesized as for the
phosphodiester oligonucleotides except the standard
oxidation bottle was replaced by 0.2 M solution of 3H-1,2-
benzodithiole-3-one 1,1-dioxide in acetonitrile for the
stepwise thiation of the phosphite linkages. The thiation
25 wait step was increased to 68 sec and was followed by the
capping step. After cleavage from the CPG column and
deblocking in concentrated ammonium hydroxide at 55°C (18
h), the oligonucleotides were purified by precipitating
twice with 2.5 volumes of ethanol from a 0.5 M NaCl
30 solution. Phosphinate oligonucleotides are prepared as
described in U.S. Patent 5,508,270, herein incorporated by
reference.

Alkyl phosphonate oligonucleotides are prepared as
described in U.S. Patent 4,469,863, herein incorporated by
35 reference.

3'-Deoxy-3'-methylene phosphonate oligonucleotides are prepared as described in U.S. Patents 5,610,289 or 5,625,050, herein incorporated by reference.

Phosphoramidite oligonucleotides are prepared as described in U.S. Patent, 5,256,775 or U.S. Patent 5,366,878, herein incorporated by reference.

Alkylphosphonothioate oligonucleotides are prepared as described in published PCT applications PCT/US94/00902 and PCT/US93/06976 (published as WO 94/17093 and WO 94/02499, respectively), herein incorporated by reference.

3'-Deoxy-3'-amino phosphoramidate oligonucleotides are prepared as described in U.S. Patent 5,476,925, herein incorporated by reference.

Phosphotriester oligonucleotides are prepared as described in U.S. Patent 5,023,243, herein incorporated by reference.

Borano phosphate oligonucleotides are prepared as described in U.S. Patents 5,130,302 and 5,177,198, both herein incorporated by reference.

Example 3

Oligonucleoside Synthesis

Methylenemethylimino linked oligonucleosides, also identified as MMI linked oligonucleosides, methylenedimethylhydrazo linked oligonucleosides, also identified as MDH linked oligonucleosides, and methylenecarbonylamino linked oligonucleosides, also identified as amide-3 linked oligonucleosides, and methyleneaminocarbonyl linked oligonucleosides, also identified as amide-4 linked oligonucleosides, as well as mixed backbone compounds having, for instance, alternating MMI and P=O or P=S linkages are prepared as described in U.S. Patents 5,378,825, 5,386,023, 5,489,677, 5,602,240 and 5,610,289, all of which are herein incorporated by reference.

Formacetal and thioformacetal linked oligonucleosides are prepared as described in U.S. Patents 5,264,562 and 5,264,564, herein incorporated by reference.

Ethylene oxide linked oligonucleosides are prepared as described in U.S. Patent 5,223,618, herein incorporated by reference.

5 **Example 4**

PNA Synthesis

Peptide nucleic acids (PNAs) are prepared in accordance with any of the various procedures referred to in Peptide Nucleic Acids (PNA): Synthesis, Properties and
10 Potential Applications, *Bioorganic & Medicinal Chemistry*, **1996**, *4*, 5-23. They may also be prepared in accordance with U.S. Patents 5,539,082, 5,700,922, and 5,719,262, herein incorporated by reference.

15 **Example 5**

Synthesis of Chimeric Oligonucleotides

Chimeric oligonucleotides, oligonucleosides or mixed oligonucleotides/oligonucleosides of the invention can be of several different types. These include a first type
20 wherein the "gap" segment of linked nucleosides is positioned between 5' and 3' "wing" segments of linked nucleosides and a second "open end" type wherein the "gap" segment is located at either the 3' or the 5' terminus of the oligomeric compound. Oligonucleotides of the first
25 type are also known in the art as "gapmers" or gapped oligonucleotides. Oligonucleotides of the second type are also known in the art as "hemimers" or "wingmers".

[2'-O-Me]--[2'-deoxy]--[2'-O-Me] Chimeric
30 **Phosphorothioate Oligonucleotides**

Chimeric oligonucleotides having 2'-O-alkyl phosphorothioate and 2'-deoxy phosphorothioate oligo-nucleotide segments are synthesized using an Applied Biosystems automated DNA synthesizer Model 380B, as above.
35 Oligonucleotides are synthesized using the automated

synthesizer and 2'-deoxy-5'-dimethoxytrityl-3'-O-phosphor-
amidite for the DNA portion and 5'-dimethoxytrityl-2'-O-
methyl-3'-O-phosphoramidite for 5' and 3' wings. The
standard synthesis cycle is modified by increasing the wait

5   step after the delivery of tetrazole and base to 600 s
repeated four times for RNA and twice for 2'-O-methyl. The
fully protected oligonucleotide is cleaved from the support
and the phosphate group is deprotected in 3:1
ammonia/ethanol at room temperature overnight then

10   lyophilized to dryness. Treatment in methanolic ammonia
for 24 hrs at room temperature is then done to deprotect
all bases and sample was again lyophilized to dryness. The
pellet is resuspended in 1M TBAF in THF for 24 hrs at room
temperature to deprotect the 2' positions. The reaction is

15   then quenched with 1M TEAA and the sample is then reduced
to 1/2 volume by rotovac before being desalted on a G25
size exclusion column. The oligo recovered is then
analyzed spectrophotometrically for yield and for purity by
capillary electrophoresis and by mass spectrometry.

20

[2'-O-(2-Methoxyethyl)]--[2'-deoxy]--[2'-O-(Methoxyethyl)] Chimeric Phosphorothioate Oligonucleotides

[2'-O-(2-methoxyethyl)]--[2'-deoxy]--[-2'-O-(methoxy-
25   ethyl)] chimeric phosphorothioate oligonucleotides were
prepared as per the procedure above for the 2'-O-methyl
chimeric oligonucleotide, with the substitution of 2'-O-
(methoxyethyl) amidites for the 2'-O-methyl amidites.

30   ### [2'-O-(2-Methoxyethyl)Phosphodiester]--[2'-deoxy Phosphorothioate]--[2'-O-(2-Methoxyethyl) Phosphodiester] Chimeric Oligonucleotides

[2'-O-(2-methoxyethyl phosphodiester]--[2'-deoxy phos-
phorothioate]--[2'-O-(methoxyethyl) phosphodiester]
35   chimeric oligonucleotides are prepared as per the above
procedure for the 2'-O-methyl chimeric oligonucleotide with

the substitution of 2'-O-(methoxyethyl) amidites for the
2'-O-methyl amidites, oxidization with iodine to generate
the phosphodiester internucleotide linkages within the wing
portions of the chimeric structures and sulfurization
5   utilizing 3,H-1,2 benzodithiole-3-one 1,1 dioxide (Beaucage
Reagent) to generate the phosphorothioate internucleotide
linkages for the center gap.

Other chimeric oligonucleotides, chimeric oligonucleo-
sides and mixed chimeric oligonucleotides/oligonucleosides
10 are synthesized according to United States patent
5,623,065, herein incorporated by reference.

Example 6

Oligonucleotide Isolation

15   After cleavage from the controlled pore glass column
(Applied Biosystems) and deblocking in concentrated
ammonium hydroxide at 55°C for 18 hours, the
oligonucleotides or oligonucleosides are purified by
precipitation twice out of 0.5 M NaCl with 2.5 volumes
20 ethanol. Synthesized oligonucleotides were analyzed by
polyacrylamide gel electrophoresis on denaturing gels and
judged to be at least 85% full length material. The
relative amounts of phosphorothioate and phosphodiester
linkages obtained in synthesis were periodically checked by
25 ^{31}P nuclear magnetic resonance spectroscopy, and for some
studies oligonucleotides were purified by HPLC, as
described by Chiang et al., *J. Biol. Chem.* **1991**, *266*,
18162-18171. Results obtained with HPLC-purified material
were similar to those obtained with non-HPLC purified
30 material.

Example 7

Oligonucleotide Synthesis - 96 Well Plate Format

Oligonucleotides were synthesized via solid phase
35 P(III) phosphoramidite chemistry on an automated
synthesizer capable of assembling 96 sequences

simultaneously in a standard 96 well format.
Phosphodiester internucleotide linkages were afforded by
oxidation with aqueous iodine. Phosphorothioate
internucleotide linkages were generated by sulfurization

5 utilizing 3,H-1,2 benzodithiole-3-one 1,1 dioxide (Beaucage
Reagent) in anhydrous acetonitrile. Standard base-
protected beta-cyanoethyldiisopropyl phosphoramidites were
purchased from commercial vendors (e.g. PE-Applied
Biosystems, Foster City, CA, or Pharmacia, Piscataway, NJ).

10 Non-standard nucleosides are synthesized as per known
literature or patented methods. They are utilized as base
protected beta-cyanoethyldiisopropyl phosphoramidites.

Oligonucleotides were cleaved from support and
deprotected with concentrated NH_4OH at elevated temperature

15 (55-60°C) for 12-16 hours and the released product then
dried in vacuo. The dried product was then re-suspended in
sterile water to afford a master plate from which all
analytical and test plate samples are then diluted
utilizing robotic pipettors.

20

Example 8

Oligonucleotide Analysis - 96 Well Plate Format

The concentration of oligonucleotide in each well was
assessed by dilution of samples and UV absorption

25 spectroscopy. The full-length integrity of the individual
products was evaluated by capillary electrophoresis (CE) in
either the 96 well format (Beckman P/ACE™ MDQ) or, for
individually prepared samples, on a commercial CE apparatus
(e.g., Beckman P/ACE™ 5000, ABI 270). Base and backbone

30 composition was confirmed by mass analysis of the compounds
utilizing electrospray-mass spectroscopy. All assay test
plates were diluted from the master plate using single and
multi-channel robotic pipettors. Plates were judged to be
acceptable if at least 85% of the compounds on the plate

35 were at least 85% full length.

Example 9

Cell culture and oligonucleotide treatment

The effect of antisense compounds on target nucleic acid expression can be tested in any of a variety of cell types provided that the target nucleic acid is present at measurable levels. This can be routinely determined using, for example, PCR or Northern blot analysis. The following 4 cell types are provided for illustrative purposes, but other cell types can be routinely used, provided that the target is expressed in the cell type chosen. This can be readily determined by methods routine in the art, for example Northern blot analysis, Ribonuclease protection assays, or RT-PCR.

T-24 cells:

The human transitional cell bladder carcinoma cell line T-24 was obtained from the American Type Culture Collection (ATCC) (Manassas, VA). T-24 cells were routinely cultured in complete McCoy's 5A basal media (Gibco/Life Technologies, Gaithersburg, MD) supplemented with 10% fetal calf serum (Gibco/Life Technologies, Gaithersburg, MD), penicillin 100 units per mL, and streptomycin 100 micrograms per mL (Gibco/Life Technologies, Gaithersburg, MD). Cells were routinely passaged by trypsinization and dilution when they reached 90% confluence. Cells were seeded into 96-well plates (Falcon-Primaria #3872) at a density of 7000 cells/well for use in RT-PCR analysis.

For Northern blotting or other analysis, cells may be seeded onto 100 mm or other standard tissue culture plates and treated similarly, using appropriate volumes of medium and oligonucleotide.

A549 cells:

The human lung carcinoma cell line A549 was obtained from the American Type Culture Collection (ATCC) (Manassas, VA). A549 cells were routinely cultured in DMEM basal

media (Gibco/Life Technologies, Gaithersburg, MD)
supplemented with 10% fetal calf serum (Gibco/Life
Technologies, Gaithersburg, MD), penicillin 100 units per
mL, and streptomycin 100 micrograms per mL (Gibco/Life
5   Technologies, Gaithersburg, MD). Cells were routinely
passaged by trypsinization and dilution when they reached
90% confluence.

NHDF cells:

10   Human neonatal dermal fibroblast (NHDF) were obtained
from the Clonetics Corporation (Walkersville MD). NHDFs
were routinely maintained in Fibroblast Growth Medium
(Clonetics Corporation, Walkersville MD) supplemented as
recommended by the supplier. Cells were maintained for up
15   to 10 passages as recommended by the supplier.

HEK cells:

          Human embryonic keratinocytes (HEK) were obtained from
the Clonetics Corporation (Walkersville MD). HEKs were
20   routinely maintained in Keratinocyte Growth Medium
(Clonetics Corporation, Walkersville MD) formulated as
recommended by the supplier. Cells were routinely
maintained for up to 10 passages as recommended by the
supplier.

25

Treatment with antisense compounds:
          When cells reached 80% confluency, they were treated
with oligonucleotide. For cells grown in 96-well plates,
wells were washed once with 200 µL OPTI-MEM™-1 reduced-
30   serum medium (Gibco BRL) and then treated with 130 µL of
OPTI-MEM™-1 containing 3.75 µg/mL LIPOFECTIN™ (Gibco BRL)
and the desired concentration of oligonucleotide. After 4-
7 hours of treatment, the medium was replaced with fresh
medium. Cells were harvested 16-24 hours after
35   oligonucleotide treatment.

The concentration of oligonucleotide used varies from cell line to cell line. To determine the optimal oligonucleotide concentration for a particular cell line, the cells are treated with a positive control

5   oligonucleotide at a range of concentrations. For human cells the positive control oligonucleotide is ISIS 13920, **TCC**GTCATCGCT**CCTCAGGG**, SEQ ID NO: 1, a 2'-O-methoxyethyl gapmer (2'-O-methoxyethyls shown in bold) with a phosphorothioate backbone which is targeted to human H-ras.

10 For mouse or rat cells the positive control oligonucleotide is ISIS 15770, **ATGCA**TTCTGCCCCC**AAGGA**, SEQ ID NO: 2, a 2'-O-methoxyethyl gapmer (2'-O-methoxyethyls shown in bold) with a phosphorothioate backbone which is targeted to both mouse and rat c-raf. The concentration of positive control

15 oligonucleotide that results in 80% inhibition of c-Ha-ras (for ISIS 13920) or c-raf (for ISIS 15770) mRNA is then utilized as the screening concentration for new oligonucleotides in subsequent experiments for that cell line. If 80% inhibition is not achieved, the lowest

20 concentration of positive control oligonucleotide that results in 60% inhibition of H-ras or c-raf mRNA is then utilized as the oligonucleotide screening concentration in subsequent experiments for that cell line. If 60% inhibition is not achieved, that particular cell line is

25 deemed as unsuitable for oligonucleotide transfection experiments.

Example 10

Analysis of oligonucleotide inhibition of superoxide

30 **dismutase 1, soluble expression**

Antisense modulation of superoxide dismutase 1, soluble expression can be assayed in a variety of ways known in the art. For example, superoxide dismutase 1, soluble mRNA levels can be quantitated by, e.g., Northern

35 blot analysis, competitive polymerase chain reaction (PCR), or real-time PCR (RT-PCR). Real-time quantitative PCR is

presently preferred. RNA analysis can be performed on total cellular RNA or poly(A)+ mRNA. Methods of RNA isolation are taught in, for example, Ausubel, *F.M. et al.,* *Current Protocols in Molecular Biology,* Volume 1, pp.

5 4.1.1-4.2.9 and 4.5.1-4.5.3, John Wiley & Sons, Inc., **1993.** Northern blot analysis is routine in the art and is taught in, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology,* Volume 1, pp. 4.2.1-4.2.9, John Wiley & Sons, Inc., **1996.** Real-time quantitative (PCR) can be

10 conveniently accomplished using the commercially available ABI PRISM™ 7700 Sequence Detection System, available from PE-Applied Biosystems, Foster City, CA and used according to manufacturer's instructions.

Protein levels of superoxide dismutase 1, soluble can
15 be quantitated in a variety of ways well known in the art, such as immunoprecipitation, Western blot analysis (immunoblotting), ELISA or fluorescence-activated cell sorting (FACS). Antibodies directed to superoxide dismutase 1, soluble can be identified and obtained from a
20 variety of sources, such as the MSRS catalog of antibodies (Aerie Corporation, Birmingham, MI), or can be prepared via conventional antibody generation methods. Methods for preparation of polyclonal antisera are taught in, for example, Ausubel, F.M. et al., *Current Protocols in*
25 *Molecular Biology,* Volume 2, pp. 11.12.1-11.12.9, John Wiley & Sons, Inc., **1997.** Preparation of monoclonal antibodies is taught in, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology,* Volume 2, pp. 11.4.1-11.11.5, John Wiley & Sons, Inc., **1997.**
30 Immunoprecipitation methods are standard in the art and can be found at, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology,* Volume 2, pp. 10.16.1-10.16.11, John Wiley & Sons, Inc., **1998.** Western blot (immunoblot) analysis is standard in the art and can

be found at, for example, Ausubel, F.M. et al., *Current*

Protocols in Molecular Biology, Volume 2, pp. 10.8.1-

10.8.21, John Wiley & Sons, Inc., **1997**. Enzyme-linked

immunosorbent assays (ELISA) are standard in the art and

5   can be found at, for example, Ausubel, F.M. et al., *Current*

Protocols in Molecular Biology, Volume 2, pp. 11.2.1-

11.2.22, John Wiley & Sons, Inc., **1991**.

Example 11

10   **Poly(A)+ mRNA isolation**

Poly(A)+ mRNA was isolated according to Miura et al.,

Clin. Chem., **1996**, *42*, 1758-1764. Other methods for

poly(A)+ mRNA isolation are taught in, for example,

Ausubel, F.M. et al., *Current Protocols in Molecular*

15   *Biology*, Volume 1, pp. 4.5.1-4.5.3, John Wiley & Sons,

Inc., **1993**. Briefly, for cells grown on 96-well plates,

growth medium was removed from the cells and each well was

washed with 200 µL cold PBS. 60 µL lysis buffer (10 mM

Tris-HCl, pH 7.6, 1 mM EDTA, 0.5 M NaCl, 0.5% NP-40, 20 mM

20   vanadyl-ribonucleoside complex) was added to each well, the

plate was gently agitated and then incubated at room

temperature for five minutes. 55 µL of lysate was

transferred to Oligo d(T) coated 96-well plates (AGCT Inc.,

Irvine CA). Plates were incubated for 60 minutes at room

25   temperature, washed 3 times with 200 µL of wash buffer (10

mM Tris-HCl pH 7.6, 1 mM EDTA, 0.3 M NaCl). After the

final wash, the plate was blotted on paper towels to remove

excess wash buffer and then air-dried for 5 minutes. 60 µL

of elution buffer (5 mM Tris-HCl pH 7.6), preheated to 70°C

30   was added to each well, the plate was incubated on a 90°C

hot plate for 5 minutes, and the eluate was then

transferred to a fresh 96-well plate.

Cells grown on 100 mm or other standard plates may be treated similarly, using appropriate volumes of all solutions.

5   **Example 12**

Total RNA Isolation

Total RNA was isolated using an RNEASY 96™ kit and buffers purchased from Qiagen Inc. (Valencia CA) following the manufacturer's recommended procedures. Briefly, for
10   cells grown on 96-well plates, growth medium was removed from the cells and each well was washed with 200 μL cold PBS. 100 μL Buffer RLT was added to each well and the plate vigorously agitated for 20 seconds. 100 μL of 70% ethanol was then added to each well and the contents mixed by
15   pipetting three times up and down. The samples were then transferred to the RNEASY 96™ well plate attached to a QIAVAC™ manifold fitted with a waste collection tray and attached to a vacuum source. Vacuum was applied for 15 seconds. 1 mL of Buffer RW1 was added to each well of the
20   RNEASY 96™ plate and the vacuum again applied for 15 seconds. 1 mL of Buffer RPE was then added to each well of the RNEASY 96™ plate and the vacuum applied for a period of 15 seconds. The Buffer RPE wash was then repeated and the vacuum was applied for an additional 10 minutes. The plate
25   was then removed from the QIAVAC™ manifold and blotted dry on paper towels. The plate was then re-attached to the QIAVAC™ manifold fitted with a collection tube rack containing 1.2 mL collection tubes. RNA was then eluted by pipetting 60 μL water into each well, incubating 1 minute,
30   and then applying the vacuum for 30 seconds. The elution step was repeated with an additional 60 μL water.

The repetitive pipetting and elution steps may be automated using a QIAGEN Bio-Robot 9604 (Qiagen, Inc., Valencia CA). Essentially, after lysing of the cells on

the culture plate, the plate is transferred to the robot
deck where the pipetting, DNase treatment and elution steps
are carried out.

5   **Example 13**

    Real-time Quantitative PCR Analysis of superoxide dismutase
    1, soluble mRNA Levels

        Quantitation of superoxide dismutase 1, soluble mRNA
    levels was determined by real-time quantitative PCR using
10 the ABI PRISM™ 7700 Sequence Detection System (PE-Applied
    Biosystems, Foster City, CA) according to manufacturer's
    instructions. This is a closed-tube, non-gel-based,
    fluorescence detection system which allows high-throughput
    quantitation of polymerase chain reaction (PCR) products in
15 real-time. As opposed to standard PCR, in which
    amplification products are quantitated after the PCR is
    completed, products in real-time quantitative PCR are
    quantitated as they accumulate. This is accomplished by
    including in the PCR reaction an oligonucleotide probe that
20 anneals specifically between the forward and reverse PCR
    primers, and contains two fluorescent dyes. A reporter dye
    (e.g., JOE, FAM, or VIC, obtained from either Operon
    Technologies Inc., Alameda, CA or PE-Applied Biosystems,
    Foster City, CA) is attached to the 5' end of the probe and
25 a quencher dye (e.g., TAMRA, obtained from either Operon
    Technologies Inc., Alameda, CA or PE-Applied Biosystems,
    Foster City, CA) is attached to the 3' end of the probe.
    When the probe and dyes are intact, reporter dye emission
    is quenched by the proximity of the 3' quencher dye.
30 During amplification, annealing of the probe to the target
    sequence creates a substrate that can be cleaved by the 5'-
    exonuclease activity of Taq polymerase. During the
    extension phase of the PCR amplification cycle, cleavage of
    the probe by Taq polymerase releases the reporter dye from
35 the remainder of the probe (and hence from the quencher
    moiety) and a sequence-specific fluorescent signal is

generated. With each cycle, additional reporter dye
molecules are cleaved from their respective probes, and the
fluorescence intensity is monitored at regular intervals by
laser optics built into the ABI PRISM™ 7700 Sequence

5   Detection System. In each assay, a series of parallel
reactions containing serial dilutions of mRNA from
untreated control samples generates a standard curve that
is used to quantitate the percent inhibition after
antisense oligonucleotide treatment of test samples.

10   Prior to quantitative PCR analysis, primer-probe sets
specific to the target gene being measured are evaluated
for their ability to be "multiplexed" with a GAPDH
amplification reaction. In multiplexing, both the target
gene and the internal standard gene GAPDH are amplified

15   concurrently in a single sample. In this analysis, mRNA
isolated from untreated cells is serially diluted. Each
dilution is amplified in the presence of primer-probe sets
specific for GAPDH only, target gene only ("single-
plexing"), or both (multiplexing). Following PCR

20   amplification, standard curves of GAPDH and target mRNA
signal as a function of dilution are generated from both
the single-plexed and multiplexed samples. If both the
slope and correlation coefficient of the GAPDH and target
signals generated from the multiplexed samples fall within

25   10% of their corresponding values generated from the
single-plexed samples, the primer-probe set specific for
that target is deemed multiplexable. Other methods of PCR
are also known in the art.

        PCR reagents were obtained from PE-Applied Biosystems,

30   Foster City, CA. RT-PCR reactions were carried out by
adding 25 μL PCR cocktail (1x TAQMAN™ buffer A, 5.5 mM
$MgCl_2$, 300 μM each of dATP, dCTP and dGTP, 600 μM of dUTP,
100 nM each of forward primer, reverse primer, and probe,
20 Units RNAse inhibitor, 1.25 Units AMPLITAQ GOLD™, and

35   12.5 Units MuLV reverse transcriptase) to 96 well plates
containing 25 μL total RNA solution. The RT reaction was

carried out by incubation for 30 minutes at 48°C. Following

a 10 minute incubation at 95°C to activate the AMPLITAQ

GOLD™, 40 cycles of a two-step PCR protocol were carried

out: 95°C for 15 seconds (denaturation) followed by 60°C for

5 1.5 minutes (annealing/extension).

Gene target quantities obtained by real time RT-PCR

are normalized using either the expression level of GAPDH,

a gene whose expression is constant, or by quantifying

total RNA using RiboGreen™ (Molecular Probes, Inc. Eugene,

10 OR). GAPDH expression is quantified by real time RT-PCR, by

being run simultaneously with the target, multiplexing, or

separately. Total RNA is quantified using RiboGreen™ RNA

quantification reagent from Molecular Probes. Methods of

RNA quantification by RiboGreen™ are taught in Jones, L.J.,

15 et al, *Analytical Biochemistry*, **1998**, *265*, 368-374.

In this assay, 175 µL of RiboGreen™ working reagent

(RiboGreen™ reagent diluted 1:2865 in 10mM Tris-HCl, 1 mM

EDTA, pH 7.5) is pipetted into a 96-well plate containing

25uL purified, cellular RNA. The plate is read in a

20 CytoFluor 4000 (PE Applied Biosystems) with excitation at

480nm and emission at 520nm.

Probes and primers to human superoxide dismutase 1,

soluble were designed to hybridize to a human superoxide

dismutase 1, soluble sequence, using published sequence

25 information (GenBank accession number X02317, incorporated

herein as SEQ ID NO:3). For human superoxide dismutase 1,

soluble the PCR primers were:

forward primer: CGTGGCCTAGCGAGTTATGG (SEQ ID NO: 4)

reverse primer: GAAATTGATGATGCCCTGCA (SEQ ID NO: 5) and the

30 PCR probe was: FAM-ACGAAGGCCGTGTGCGTGCTG-TAMRA

(SEQ ID NO: 6) where FAM (PE-Applied Biosystems, Foster

City, CA) is the fluorescent reporter dye) and TAMRA (PE-

Applied Biosystems, Foster City, CA) is the quencher dye.

For human GAPDH the PCR primers were:

35 forward primer: GAAGGTGAAGGTCGGAGTC (SEQ ID NO: 7)

reverse primer: GAAGATGGTGATGGGATTTC (SEQ ID NO: 8) and the

PCR probe was: 5' JOE-CAAGCTTCCCGTTCTCAGCC- TAMRA 3' (SEQ ID NO: 9) where JOE (PE-Applied Biosystems, Foster City, CA) is the fluorescent reporter dye) and TAMRA (PE-Applied Biosystems, Foster City, CA) is the quencher dye.

5

Example 14

Northern blot analysis of superoxide dismutase 1, soluble mRNA levels

Eighteen hours after antisense treatment, cell

10 monolayers were washed twice with cold PBS and lysed in 1 mL RNAZOL™ (TEL-TEST "B" Inc., Friendswood, TX). Total RNA was prepared following manufacturer's recommended protocols. Twenty micrograms of total RNA was fractionated by electrophoresis through 1.2% agarose gels containing

15 1.1% formaldehyde using a MOPS buffer system (AMRESCO, Inc. Solon, OH). RNA was transferred from the gel to HYBOND™-N+ nylon membranes (Amersham Pharmacia Biotech, Piscataway, NJ) by overnight capillary transfer using a Northern/Southern Transfer buffer system (TEL-TEST "B"

20 Inc., Friendswood, TX). RNA transfer was confirmed by UV visualization. Membranes were fixed by UV cross-linking using a STRATALINKER™ UV Crosslinker 2400 (Stratagene, Inc, La Jolla, CA) and then robed using QUICKHYB™ hybridization solution (Stratagene, La Jolla, CA) using manufacturer's

25 recommendations for stringent conditions.

To detect human superoxide dismutase 1, soluble, a human superoxide dismutase 1, soluble specific probe was prepared by PCR using the forward primer CGTGGCCTAGCGAGTTATGG (SEQ ID NO: 4) and the reverse primer

30 GAAATTGATGATGCCCTGCA (SEQ ID NO: 5). To normalize for variations in loading and transfer efficiency membranes were stripped and probed for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA (Clontech, Palo Alto, CA).

35

Hybridized membranes were visualized and quantitated
using a PHOSPHORIMAGER™ and IMAGEQUANT™ Software V3.3
(Molecular Dynamics, Sunnyvale, CA). Data was normalized
to GAPDH levels in untreated controls.

5

Example 15

**Antisense inhibition of human superoxide dismutase 1,
soluble expression by chimeric phosphorothioate
oligonucleotides having 2'-MOE wings and a deoxy gap**

10   In accordance with the present invention, a series of
oligonucleotides was designed to target different regions
of the human superoxide dismutase 1, soluble RNA, using
published sequences (GenBank accession number X02317,
incorporated herein as SEQ ID NO: 3, genomic sequence
15 representing residues 15001-26000 of GenBank accession
number AP000213.1, incorporated herein as SEQ ID NO: 10,
GenBank accession number AI085992, an EST suggesting a
splice variant of superoxide dismutase 1, soluble lacking
exon 2, the complement of which is incorporated herein as
20 SEQ ID NO: 11, and GenBank accession number N28535 which
extends SEQ ID NO:3 in the 5' direction, incorporated
herein as SEQ ID NO: 12). The oligonucleotides are shown
in Table 1. ''Target site'' indicates the first (5'-most)
nucleotide number on the particular target sequence to
25 which the oligonucleotide binds. All compounds in Table 1
are chimeric oligonucleotides ("gapmers") 20 nucleotides in
length, composed of a central "gap" region consisting of
ten 2'-deoxynucleotides, which is flanked on both sides (5'
and 3' directions) by five-nucleotide "wings". The wings
30 are composed of 2'-methoxyethyl (2'-MOE)nucleotides. The
internucleoside (backbone) linkages are phosphorothioate
(P=S) throughout the oligonucleotide. All cytidine
residues are 5-methylcytidines. The compounds were
analyzed for their effect on human superoxide dismutase 1,
35 soluble mRNA levels by quantitative real-time PCR as

described in other examples herein. Data are averages from
two experiments. If present, "N.D." indicates "no data".

Table 1

Inhibition of human superoxide dismutase 1, soluble mRNA

levels by chimeric phosphorothioate oligonucleotides having

2'-MOE wings and a deoxy gap

ISIS #	REGION	TARGET SEQ ID NO	TARGET SITE	SEQUENCE	%INHIB	SEQ ID NO
146143	Coding	3	73	tcagcacgcacacggccttc	95	13
146144	Coding	3	78	gcccttcagcacgcacacgg	0	14
146145	Coding	3	81	gtcgcccttcagcacgcaca	97	15
150437	5'UTR	3	14	cgaggactgcaacggaaacc	0	16
150438	5'UTR	3	19	ggttccgaggactgcaacgg	87	17
150439	5'UTR	3	23	tcctggttccgaggactgca	84	18
150440	5'UTR	3	27	gaggtcctggttccgaggac	0	19
150441	5'UTR	3	38	taggccacgccgaggtcctg	84	20
150442	Start Codon	3	53	gtcgccataactcgctaggc	4	21
150443	Coding	3	96	gccctgcactgggccgtcgc	94	22
150444	Coding	3	106	aattgatgatgccctgcact	62	23
150445	Coding	3	135	cactggtccattactttcct	91	24
150446	Coding	3	142	acaccttcactggtccatta	93	25
150447	Coding	3	144	ccacaccttcactggtccat	0	26
150448	Coding	3	161	agtcctttaatgcttcccca	86	27
150449	Coding	3	173	aggccttcagtcagtccttt	29	28
150450	Coding	3	174	caggccttcagtcagtcctt	90	29
150451	Coding	3	205	tatctccaaactcatgaaca	68	30
150452	Coding	3	212	gctgtattatctccaaactc	90	31
150453	Coding	3	221	gtacagcctgctgtattatc	69	32
150454	Coding	3	304	tgcccaagtctccaacatgc	89	33
150455	Coding	3	309	cacattgcccaagtctccaa	22	34
150456	Coding	3	335	tcggccacaccatctttgtc	85	35
150457	Coding	3	337	catcggccacaccatctttg	94	36
150458	Coding	3	340	acacatcggccacaccatct	86	37
150459	Coding	3	343	tagacacatcggccacacca	87	38
150460	Coding	3	404	accaccagtgtgcggccaat	21	39
150461	Coding	3	409	catggaccaccagtgtgcgg	75	40
150462	Coding	3	410	tcatggaccaccagtgtgcg	59	41
150463	Coding	3	504	ggcgatcccaattacaccac	94	42
150464	Stop Codon	3	517	ggaatgtttattgggcgatc	91	43
150465	3'UTR	3	535	cctcagactacatccaaggg	37	44

150466	3'UTR	3	556	gataacagatgagttaaggg	61	45
150467	3'UTR	3	620	cacaattacacttttaagat	21	46
150468	3'UTR	3	625	agtcacacaattacacttt	0	47
150469	3'UTR	3	658	ctcactacaggtactttaaa	50	48
150470	3'UTR	3	667	aatcagtttctcactacagg	0	49
150471	3'UTR	3	670	ataaatcagtttctcactac	46	50
150472	3'UTR	3	671	cataaatcagtttctcacta	47	51
150473	3'UTR	3	686	aatcttccaagtgatcataa	55	52
150474	3'UTR	3	691	atacaaatcttccaagtgat	48	53
150475	3'UTR	3	707	tgagttttataaaactatac	2	54
150476	3'UTR	3	710	aactgagttttataaaacta	23	55
150477	3'UTR	3	721	acagacatttttaactgagtt	49	56
150478	3'UTR	3	727	attgaaacagacatttttaac	45	57
150479	3'UTR	3	729	tcattgaaacagacatttta	41	58
150480	3'UTR	3	736	atacaggtcattgaaacaga	66	59
150481	3'UTR	3	761	ccatctgtgatttaagtctg	58	60
150482	3'UTR	3	769	tttaatacccatctgtgatt	50	61
150483	3'UTR	3	771	agtttaatacccatctgtga	43	62
150484	3'UTR	3	787	caaagaaattctgacaagtt	44	63
150485	3'UTR	3	795	ttgaatgacaaagaaattct	3	64
150486	3'UTR	3	801	acaggcttgaatgacaaaga	0	65
150487	3'UTR	3	805	attcacaggcttgaatgaca	0	66
150488	3'UTR	3	812	ggtttttattcacaggcttg	53	67
150489	3'UTR	3	814	agggttttattcacaggct	34	68
150490	3'UTR	3	818	atacagggttttttattcaca	63	69
150491	3'UTR	3	820	ccatacagggttttttattca	44	70
150492	3'UTR	3	825	aagtgccatacagggttttt	40	71
150493	3'UTR	3	829	taataagtgccatacagggt	27	72
150494	3'UTR	3	832	tcataataagtgccatacag	0	73
150495	3'UTR	3	833	ctcataataagtgccataca	52	74
150496	3'UTR	3	835	gcctcataataagtgccata	47	75
150497	3'UTR	3	843	ttttaatagcctcataataa	31	76
150498	3'UTR	3	849	ggattcttttaatagcctca	38	77
150499	Intron: Exon Junction	10	790	cagcccttgccttctgctcg	86	78
150500	Intron 1	10	3845	agtagctgggactacaggcg	0	79
150501	Intron 1	10	4738	cattactttcctttaagaaa	63	80
150502	Intron 2	10	6248	aagatcactaaatgcaactt	57	81
150503	Intron 2	10	7023	caggagaatcgcttgaacct	9	82
150504	Intron: Exon Junction	10	7397	ctggtacagcctatttataa	65	83
150505	Intron 3	10	8053	gcttcacgtctacacactaa	28	84
150506	Intron: Exon Junction	10	8206	tccaacatgcctaataatga	36	85
150507	mRNA	11	30	tggtacagccttctgctcga	0	86
150508	5'UTR	12	20	taggccagacctccgcgcct	0	87

150509	5'UTR	12	26	actttataggccagacctcc	0	88
150510	5'UTR	12	56	gacgcaaaccagcaccccgt	29	89
150511	5'UTR	12	73	acgctgcaggagactacgac	81	90

　　　　As shown in Table 1, SEQ ID NOs 13, 15, 17, 18, 20,
22, 23, 24, 25, 27, 29, 30, 31, 32, 33, 35, 36, 37, 38, 40,
41, 42, 43, 45, 48, 52, 59, 60, 61, 67, 69, 74, 78, 80, 81,
5　83 and 90 demonstrated at least 50% inhibition of human
superoxide dismutase 1, soluble expression in this assay
and are therefore preferred. The target sites to which
these preferred sequences are complementary are herein
referred to as "active sites" and are therefore preferred
10　sites for targeting by compounds of the present invention.

Example 16

**Western blot analysis of superoxide dismutase 1, soluble
protein levels**

15　　　　Western blot analysis (immunoblot analysis) was
carried out using standard methods. Cells (A549 and rat
A10) were harvested 16-20 h after oligonucleotidetreatment,
washed once with PBS, suspended in Laemmli buffer (100
ul/well), boiled for 5 minutes and loaded on a 16% SDS-PAGE
20　gel. Superoxide dismutase 1, soluble oligonucleotides used
were ISIS 146144 and ISIS 146145. A scrambled superoxide
dismutase 1, soluble oligonucleotide was used as a negative
control, as were cells not treated with oligonucleotide
(untreated control). Gels are run for 1.5 hours at 150 V,
25　and transferred to membrane for western blotting.
Appropriate primary antibody directed to superoxide
dismutase 1, soluble was used, with a radiolabelled or
fluorescently labeled secondary antibody directed against
the primary antibody species. Bands were visualized using
30　a PHOSPHORIMAGER™ (Molecular Dynamics, Sunnyvale CA). ISIS
146144 and 146145 each inhibited production of superoxide
dismutase 1, soluble by >75%, while the scrambled control
oligonucleotide had no effect on levels superoxide
dismutase 1, soluble. The untreated control cells also

showed no reduction in levels of superoxide dismutase 1, soluble.

Example 17

5   **Chimeric phosphorothioate oligonucleotides targeted to human superoxide dismutase 1, soluble having 2'-MOE wings and a deoxy gap**

In accordance with the present invention, a second series of oligonucleotides was designed to target different
10   regions of the human superoxide dismutase 1, soluble RNA, using published sequences (GenBank accession number X02317, incorporated herein as SEQ ID NO: 3). The oligonucleotides are shown in Table 2. ''Target site'' indicates the first (5'-most) nucleotide number on the particular target sequence to which the oligonucleotide binds. All compounds
15   in Table 2 are chimeric oligonucleotides ("gapmers") 20 nucleotides in length, composed of a central "gap" region consisting of ten 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by five-nucleotide "wings". The wings are composed of 2'-methoxyethyl (2'-
20   MOE) nucleotides. The internucleoside (backbone) linkages are phosphorothioate (P=S) throughout the oligonucleotide. All cytidine residues are 5-methylcytidines.

25   **Table 2**

Chimeric phosphorothioate oligonucleotides targeted to human superoxide dismutase 1, soluble having 2'-MOE wings and a deoxy gap

ISIS #	REGION	TARGET SEQ ID NO	TARGET SITE	SEQUENCE	SEQ ID NO
331678	start	3	66	GCACACGGCCTTCGTCGCCA	91
333605	coding	3	74	TTCAGCACGCACACGGCCTT	92
333606	coding	3	76	CCTTCAGCACGCACACGGCC	93
333607	coding	3	77	CCCTTCAGCACGCACACGGC	94
333608	coding	3	79	CGCCCTTCAGCACGCACACG	95
333609	coding	3	80	TCGCCCTTCAGCACGCACAC	96
333610	coding	3	82	CGTCGCCCTTCAGCACGCAC	97
333611	coding	3	83	CCGTCGCCCTTCAGCACGCA	98

333612	coding	3	292	CAACATGCCTCTCTTCATCC	99
333613	coding	3	293	CCAACATGCCTCTCTTCATC	100
333614	coding	3	294	TCCAACATGCCTCTCTTCAT	101
333615	coding	3	295	CTCCAACATGCCTCTCTTCA	102
333616	coding	3	296	TCTCCAACATGCCTCTCTTC	103
333617	coding	3	297	GTCTCCAACATGCCTCTCTT	104
333618	coding	3	373	CTCCTGAGAGTGAGATCACA	105
333619	coding	3	374	TCTCCTGAGAGTGAGATCAC	106
333620	coding	3	436	CACCTTTGCCCAAGTCATCT	107
333621	coding	3	437	CCACCTTTGCCCAAGTCATC	108
333622	coding	3	438	TCCACCTTTGCCCAAGTCAT	109
333623	coding	3	439	TTCCACCTTTGCCCAAGTCA	110
333625	coding	3	441	ATTTCCACCTTTGCCCAAGT	111
333626	coding	3	442	CATTTCCACCTTTGCCCAAG	112
333627	coding	3	443	TCATTTCCACCTTTGCCCAA	113
333628	coding	3	444	TTCATTTCCACCTTTGCCCA	114
333629	coding	3	445	CTTCATTTCCACCTTTGCCC	115
333630	coding	3	446	TCTTCATTTCCACCTTTGCC	116
333631	coding	3	447	TTCTTCATTTCCACCTTTGC	117
333632	coding	3	448	TTTCTTCATTTCCACCTTTG	118
333633	coding	3	449	CTTTCTTCATTTCCACCTTT	119
333634	coding	3	450	ACTTTCTTCATTTCCACCTT	120
333635	coding	3	451	TACTTTCTTCATTTCCACCT	121
333636	coding	3	452	GTACTTTCTTCATTTCCACC	122
333637	coding	3	453	TGTACTTTCTTCATTTCCAC	123
333638	coding	3	454	TTGTACTTTCTTCATTTCCA	124
333639	coding	3	455	TTTGTACTTTCTTCATTTCC	125
333640	coding	3	456	CTTTGTACTTTCTTCATTTC	126
333641	coding	3	457	TCTTTGTACTTTCTTCATTT	127
333642	coding	3	458	GTCTTTGTACTTTCTTCATT	128

Example 18

Phosphorothioate oligodeoxynucleotides targeted to human superoxide dismutase 1, soluble

5   In a further embodiment of the present invention, a third series of oligonucleotides was designed to target different regions of the human superoxide dismutase 1, soluble RNA, using published sequences (GenBank accession number X02317, incorporated herein as SEQ ID NO: 3; genomic
10   sequence representing nucleotides 15001 to 26000 of GenBank accession number AP000213.1, incorporated herein as SEQ ID NO: 10). The oligonucleotides are shown in Table 3. ''Target site'' indicates the first (5'-most) nucleotide number on the particular target sequence to which the
15   oligonucleotide binds. All compounds in Table 3 are oligodeoxynucleotides 20 nucleotides in length. The internucleoside (backbone) linkages are phosphorothioate

(P=S) throughout the oligonucleotide. All cytidine
residues are 5-methylcytidines.

Table 3

5   Phosphorothioate oligodeoxynucleotides targeted to

human superoxide dismutase 1, soluble

ISIS #	REGION	TARGET SEQ ID NO	TARGET SITE	SEQUENCE	SEQ ID NO
336629	coding	3	127	CATTACTTTCCTTCTGCTCG	129
336631	coding	3	294	TCCAACATGCCTCTCTTCAT	130
336633	intron:exon	10	4738	CATTACTTTCCTTTAAGAAA	131
336635	exon:intron	10	4835	CAACACCCACCTGCTGTATT	132
336637	intron	10	7397	CTGGTACAGCCTATTTATAA	133
336639	exon:intron	10	7468	CATCTTGTTACCTCTCTTCA	134
336641	intron:exon	10	8206	TCCAACATGCCTAATAATGA	135
336643	exon	10	8324	GAAAACTTACCACCAGTGTG	136
336645	intron:exon	10	9420	TTTTCATGGACCTGTAAAAA	137
336647	3'UTR	3	849	GGATTCTTTTAATAGCCTCA	138

        In addition to targeting human superoxide dismutase 1,
10   soluble mRNA, SEQ ID NO : 130 also targets rat superoxide
dismutase 1, soluble.
        In a further embodiment of the present invention, a
fourth series of oligonucleotides was designed to target
different regions of the human superoxide dismutase 1,
15   soluble RNA, using published sequences (GenBank accession
number X02317, incorporated herein as SEQ ID NO: 3; genomic
sequence representing nucleotides 15001 to 26000 of GenBank
accession number AP000213.1, incorporated herein as SEQ ID
NO: 10). The oligonucleotides are shown in Table 4.
20   ''Target site'' indicates the first (5'-most) nucleotide
number on the particular target sequence to which the
oligonucleotide binds. All compounds in Table 4 are
oligodeoxynucleotides 15 nucleotides in length. The
internucleoside (backbone) linkages are phosphorothioate
25   (P=S) throughout the oligonucleotide. All cytidine
residues are 5-methylcytidines.

Table 4

Phosphorothioate oligodeoxynucleotides targeted to
human superoxide dismutase 1, soluble

ISIS #	REGION	TARGET SEQ ID NO	TARGET SITE	SEQUENCE	SEQ ID NO
336630	coding	3	129	TACTTTCCTTCTGCT	139
336632	coding	3	296	AACATGCCTCTCTTC	140
336634	intron:exon	10	4740	TACTTTCCTTTAAGA	141
336636	exon:intron	10	4837	CACCCACCTGCTGTA	142
336638	intron:exon	10	7399	GTACAGCCTATTTAT	143
336640	exon:intron	10	7470	CTTGTTACCTCTCTT	144
336642	intron	10	8208	AACATGCCTAATAAT	145
336644	exon:intron	10	8326	AACTTACCACCAGTG	146
336646	intron:exon	10	9422	TCATGGACCTGTAAA	147
336648	exon	10	9858	TTCTTTTAATAGCCT	148

In addition to targeting human superoxide dismutase 1, soluble mRNA, SEQ ID NO: 140 also targets rat superoxide dismutase 1, soluble.

Example 19

Antisense inhibition of rat superoxide dismutase 1, soluble expression by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap

In accordance with the present invention, a series of oligonucleotides was designed to target different regions of the rat superoxide dismutase 1, soluble RNA, using published sequences (GenBank accession number Y00404.1, incorporated herein as SEQ ID NO: 149; GenBank accession number Z21917.1, incorporated herein as SEQ ID NO: 150; GenBank accession number Z21918.1, incorporated herein as SEQ ID NO: 151; GenBank accession number X54986.1, incorporated herein as SEQ ID NO: 152; GenBank accession number Z21919.1, incorporated herein as SEQ ID NO: 153; GenBank accession number Z21920.1, incorporated herein as SEQ ID NO: 154; GenBank accession number Z21924.1, incorporated herein as SEQ ID NO: 155; GenBank accession number X55397.1, incorporated herein as SEQ ID NO: 156).

The oligonucleotides are shown in Table 5. ``Target site''
indicates the first (5'-most) nucleotide number on the
particular target sequence to which the oligonucleotide
binds. All compounds in Table 5 are chimeric

5   oligonucleotides ("gapmers") 20 nucleotides in length,
composed of a central "gap" region consisting of ten 2'-
deoxynucleotides, which is flanked on both sides (5' and 3'
directions) by five-nucleotide "wings". The wings are
composed of 2'-methoxyethyl (2'-MOE)nucleotides. The

10   internucleoside (backbone) linkages are phosphorothioate
(P=S) throughout the oligonucleotide. All cytidine
residues are 5-methylcytidines.

The compounds were analyzed for their effect on rat
superoxide dismutase 1, soluble in primary rat hepatocytes.

15   Primary rat hepatocytes were prepared from Sprague-Dawley
rats purchased from Charles River Labs (Wilmington, MA) and
were routinely cultured in DMEM, high glucose (Gibco/Life
Technologies, Gaithersburg, MD) supplemented with 10% fetal
bovine serum (Gibco/Life Technologies, Gaithersburg, MD),

20   100 units per ml penicillin, and 100 micrograms per ml
streptomycin (Gibco/Life Technologies, Gaithersburg, MD).

Cells were seeded into 96-well plates (Falcon-Primaria
#353047, BD Biosciences, Bedford, MA) at a density of 4000-
6000 cells/well for use in antisense oligonucleotide

25   transfection. For cells grown in 96-well plates, cells
were treated with 100 uL of OPTI-MEM-1 containing 2.5 ug/mL
LIPOFECTIN (Invitrogen Corporation, Carlsbad, CA) and the
desired concentration of oligonucleotide. Cells were
treated and data are obtained in triplicate. After 4 hours

30   of treatment at 37°C, the medium was replaced with fresh
medium. Cells were harvested 16-24 hours after
oligonucleotide treatment.

ISIS 18078 was used as a control oligonucleotide.
ISIS 18078 (GTGCGCGCGAGCCCGAAATC, SEQ ID NO: 157) is a

35   chimeric oligonucleotide ("gapmers") 20 nucleotides in
length, composed of a central "gap" region consisting of
ten 2'-deoxynucleotides, which is flanked on both sides (5'

and 3' directions) by five-nucleotide "wings". The wings
are composed of 2'-methoxyethyl (2'-MOE)nucleotides. The
internucleoside (backbone) linkages are phosphorothioate
(P=S) throughout the oligonucleotide. All cytidine
5 residues are 5-methylcytidines.

The compounds were analyzed for their effect on rat
superoxide dismutase 1, soluble mRNA levels in rat
hepatocytes by quantitative real-time PCR as described in
other examples herein. Probes and primers to mouse
10 superoxide dismutase 1 were designed to hybridize to a rat
superoxide dismutase 1, soluble sequence, using published
sequence information (incorporated herein as SEQ ID NO:
149). For rat superoxide dismutase 1, soluble the PCR
primers were: forward primer: TGCTGAAGGGCGACGG (SEQ ID NO:
15 159) reverse primer: GTTCACCGCTTGCCTTCTG (SEQ ID NO: 160)
and the PCR probe was: FAM- CCGGTGCAGGGCGTCATTCACTT-TAMRA
(SEQ ID NO: 161) where FAM is the fluorescent reporter dye
and TAMRA is the quencher dye. Gene target quantities
obtained by real time RT-PCR are normalized by quantifying
20 total RNA using RiboGreen™ (Molecular Probes, Inc. Eugene,
OR). In this assay, 170 µL of RiboGreen™ working reagent
(RiboGreen™ reagent diluted 1:350 in 10mM Tris-HCl, 1 mM
EDTA, pH 7.5) is pipetted into a 96-well plate containing
30 µL purified, cellular RNA. The plate is read in a
25 CytoFluor 4000 (PE Applied Biosystems) with excitation at
485nm and emission at 530nm.

Data are averages from two experiments in which
primary rat hepatocytes were treated with the antisense
oligonucleotides of the present invention at a dose of 150
30 nM. If present, "N.D." indicates "no data".

Tabl 5

Inhibition of rat superoxide dismutase 1, soluble mRNA

levels by chimeric phosphorothioate oligonucleotides having

2'-MOE wings and a deoxy gap

5

ISIS #	REGION	TARGET SEQ ID NO	TARGET SITE	SEQUENCE	% INHIB	SEQ ID NO
146137	5'UTR	149	4	CGCAGGAAACGAAGGTGCAA	82	162
146138	5'UTR	149	6	GCCGCAGGAAACGAAGGTGC	88	163
146139	5'UTR	149	49	CTCGGAACCTGGGAGAGCAA	40	164
146140	5'UTR	149	85	TTCATCGCCATGCTTCCCCG	79	165
146141	5'UTR	149	91	ACGGCCTTCATCGCCATGCT	88	166
146142	start	149	94	CACACGGCCTTCATCGCCAT	79	167
146143	coding	149	102	TCAGCACGCACACGGCCTTC	83	168
146144	coding	149	107	GCCCTTCAGCACGCACACGG	95	169
146145	coding	149	110	GTCGCCCTTCAGCACGCACA	91	170
146146	coding	149	115	GGACCGTCGCCCTTCAGCAC	97	171
146147	coding	149	143	CTGCTCGAAGTGAATGACGC	97	172
146148	coding	149	151	CTTGCCTTCTGCTCGAAGTG	89	173
146149	coding	149	156	CACCGCTTGCCTTCTGCTCG	92	174
146150	coding	149	161	TGGTTCACCGCTTGCCTTCT	78	175
146151	coding	149	166	ACAACTGGTTCACCGCTTGC	90	176
146152	coding	149	183	TAATCTGTCCTGACACCACA	83	177
146153	coding	149	188	TCCTGTAATCTGTCCTGACA	84	178
146154	coding	149	192	TTAATCCTGTAATCTGTCCT	85	179
146155	coding	149	208	CCATGCTCGCCTTCAGTTAA	80	180
146156	coding	149	217	ACATGGAACCCATGCTCGCC	82	181
146157	coding	149	225	ATTGATGGACATGGAACCCA	87	182
146158	coding	149	244	CCTTGTGTATTGTCCCCATA	86	183
146159	coding	149	249	TACAGCCTTGTGTATTGTCC	77	184
146160	coding	149	278	GTGAGGATTAAAATGAGGTC	70	185
146161	coding	149	284	CTTAGAGTGAGGATTAAAAT	69	186
146162	coding	149	289	TGTTTCTTAGAGTGAGGATT	72	187
146163	coding	149	334	TTGCCCAGGTCTCCAACATG	79	188
146164	coding	149	337	ACATTGCCCAGGTCTCCAAC	78	189
146165	coding	149	359	CACACCGTCCTTTCCAGCAG	82	190
146166	coding	149	364	TTGGCCACACCGTCCTTTCC	82	191
146167	coding	149	367	ACATTGGCCACACCGTCCTT	74	192
146168	coding	149	369	ACACATTGGCCACACCGTCC	84	193
146169	coding	149	374	AATGGACACATTGGCCACAC	82	194
146170	coding	149	379	TCTTCAATGGACACATTGGC	78	195
146171	coding	149	384	CACGATCTTCAATGGACACA	86	196
146172	coding	149	387	TCACACGATCTTCAATGGAC	76	197
146173	coding	149	389	GATCACACGATCTTCAATGG	88	198
146174	coding	149	421	CGGCCAATGATGGAATGCTC	85	199
146175	coding	149	426	TAGTACGGCCAATGATGGAA	87	200
146176	coding	149	439	TCGTGGACCACCATAGTACG	87	201
146177	coding	149	442	TTCTCGTGGACCACCATAGT	84	202
146178	coding	149	505	CGGCTTCCAGCATTTCCAGT	77	203
146179	coding	149	510	CCAAGCGGCTTCCAGCATTT	76	204
146180	coding	149	516	CACAAGCCAAGCGGCTTCCA	77	205

146181	coding	149	522	TCACACCACAAGCCAAGCGG	74	206
146182	coding	149	532	GCAATCCCAATCACACCACA	76	207
146183	coding	149	535	TGGGCAATCCCAATCACACC	82	208
146184	coding	149	539	TTATTGGGCAATCCCAATCA	87	209
146185	coding	149	553	CACATAGGGAATGTTTATTG	81	210
146186	3'UTR	149	559	TCAGACCACATAGGGAATGT	71	211
146187	3'UTR	149	563	AGACTCAGACCACATAGGGA	64	212
146188	3'UTR	149	567	TCTGAGACTCAGACCACATA	83	213
146189	3'UTR	149	583	CAGGACAGCAGATGAGTCTG	82	214
146190	3'UTR	149	586	TAGCAGGACAGCAGATGAGT	89	215
146191	3'UTR	149	592	ACAGTTTAGCAGGACAGCAG	90	216
146192	3'UTR	149	595	TCTACAGTTTAGCAGGACAG	91	217
146193	3'UTR	149	622	GATTACAGTTTAATGGTTTG	57	218
146194	intron	150	1996	TAGCGATGCAAACTGCTCTC	0	219
146195	intron	150	2002	ATAGGATAGCGATGCAAACT	38	220
146196	exon:intron	151	521	TAGGACCTACCTTGTGTATT	19	221
146197	exon:intron	152	235	TAAGACTTACCTTGTGTATT	11	222
146198	intron	152	280	ACTCTGACCCATTCATCTCA	55	223
146199	exon:intron	153	321	GCTGCTCACCTCTCTTCATC	52	224
146200	intron	153	594	TTGCTAGTGACGTGATAGTA	51	225
146201	intron	153	659	ATACAAACGGAATCTCAACT	57	226
146202	intron	153	714	CTCCAGCTCATTCAAAGAGC	21	227
146203	intron	153	743	AGTATGCAGCTCCTGATTAC	24	228
146204	intron	153	764	GAAGGCACTTCGAGGTTACG	45	229
146205	exon:intron	154	142	GGAAACTTACCACCATAGTA	46	230
146206	exon:intron	154	145	TATGGAAACTTACCACCATA	66	231
146207	intron	154	305	GCAAATTAATTCTTTACTAT	20	232
146208	intron	154	391	GTATCCTCAACTCAGATCCA	79	233
146209	intron:exon	155	154	TCTCGTGGACCTTTGAAAAG	62	234
146210	intron	156	33	AGCAGACTACTAAGTGTTTC	44	235
146211	intron	156	52	TTTTATGCTATCAGCTAAAA	27	236
146212	intron	156	69	TAAATCAATAAGCTAATTTT	9	237
146213	intron	156	84	GTTCAAATCTATTAGTAAAT	36	238
146214	intron:exon	156	187	CTCGTGGACCTTTGAAAAGA	79	239

In addition to targeting rat superoxide dismutase 1, soluble mRNA, SEQ ID Nos 168, 169 and 170 also target human superoxide dismutase 1, soluble.

As shown in Table 5, SEQ ID Nos 162, 163, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 231, 233, 234 and 239 demonstrated at least 60% inhibition of rat superoxide dismutase 1, soluble expression in this assay and are therefore preferred. More preferred are SEQ ID NOs: 217, 216, and 215. The target sites to which these preferred sequences are complementary are herein referred

to as "active sites" and are therefore preferred sites for targeting by compounds of the present invention.

Example 20

5   **Chimeric phosphorothioate oligonucleotides targeted to rat superoxide dismutase 1, soluble having 2'-MOE wings and a deoxy gap**

In a further embodiment of the present invention, a second series of oligonucleotides was designed to target
10   different regions of the rat superoxide dismutase 1, soluble RNA, using published sequences (GenBank accession number Y00404.1, incorporated herein as SEQ ID NO: 149). The oligonucleotides are shown in Table 6. ''Target site'' indicates the first (5'-most) nucleotide number on the
15   particular target sequence to which the oligonucleotide binds. All compounds in Table 6 are chimeric oligonucleotides ("gapmers") 20 nucleotides in length, composed of a central "gap" region consisting of ten 2'-deoxynucleotides, which is flanked on both sides (5' and 3'
20   directions) by five-nucleotide "wings". The wings are composed of 2'-methoxyethyl (2'-MOE)nucleotides. The internucleoside (backbone) linkages are phosphorothioate (P=S) throughout the oligonucleotide. All cytidine residues are 5-methylcytidines.
25

Table 6

Chimeric phosphorothioate oligonucleotides targeted to rat superoxide dismutase 1, soluble having 2'-MOE wings and a deoxy gap

30

ISIS #	REGION	TARGET SEQ ID NO	TARGET SITE	SEQUENCE	SEQ ID NO
195743	5'UTR	149	40	TGGGAGAGCAAAAAGCAAGG	240
195744	5'UTR	149	45	GAACCTGGGAGAGCAAAAAG	241
195745	5'UTR	149	51	GCCTCGGAACCTGGGAGAGC	242
195746	5'UTR	149	53	CGGCCTCGGAACCTGGGAGA	243
195747	5'UTR	149	55	GGCGGCCTCGGAACCTGGGA	244

195748	5'UTR	149	77	CATGCTTCCCCGGGAGACGC	245
195749	5'UTR	149	81	TCGCCATGCTTCCCCGGGAG	246
195750	5'UTR	149	83	CATCGCCATGCTTCCCCGGG	247
195751	5'UTR	149	88	GCCTTCATCGCCATGCTTCC	248
195752	coding	149	99	GCACGCACACGGCCTTCATC	249
195753	coding	149	104	CTTCAGCACGCACACGGCCT	250
195754	coding	149	145	TTCTGCTCGAAGTGAATGAC	251
195755	coding	149	149	TGCCTTCTGCTCGAAGTGAA	252
195756	coding	149	153	CGCTTGCCTTCTGCTCGAAG	253
195757	coding	149	158	TTCACCGCTTGCCTTCTGCT	254
195758	coding	149	168	CCACAACTGGTTCACCGCTT	255
195759	coding	149	170	CACCACAACTGGTTCACCGC	256
195760	coding	149	178	TGTCCTGACACCACAACTGG	257
195761	coding	149	180	TCTGTCCTGACACCACAACT	258
195762	coding	149	185	TGTAATCTGTCCTGACACCA	259
195763	coding	149	190	AATCCTGTAATCTGTCCTGA	260
195764	coding	149	194	AGTTAATCCTGTAATCTGTC	261
195765	coding	149	196	TCAGTTAATCCTGTAATCTG	262
195766	coding	149	210	ACCCATGCTCGCCTTCAGTT	263
195767	coding	149	215	ATGGAACCCATGCTCGCCTT	264
195768	coding	149	219	GGACATGGAACCCATGCTCG	265
195769	coding	149	223	TGATGGACATGGAACCCATG	266
195770	coding	149	228	CATATTGATGGACATGGAAC	267
195771	coding	149	230	CCCATATTGATGGACATGGA	268
195772	coding	149	232	TCCCCATATTGATGGACATG	269
195773	coding	149	234	TGTCCCCATATTGATGGACA	270
195774	coding	149	236	ATTGTCCCCATATTGATGGA	271
195775	coding	149	241	TGTGTATTGTCCCCATATTG	272
195776	coding	149	246	AGCCTTGTGTATTGTCCCCA	273
195777	coding	149	286	TTCTTAGAGTGAGGATTAAA	274
195778	coding	149	291	CATGTTTCTTAGAGTGAGGA	275
195779	coding	149	293	GCCATGTTTCTTAGAGTGAG	276
195780	coding	149	297	GACCGCCATGTTTCTTAGAG	277
195781	coding	149	299	TGGACCGCCATGTTTCTTAG	278
195782	coding	149	301	GCTGGACCGCCATGTTTCTT	279
195783	coding	149	303	CCGCTGGACCGCCATGTTTC	280
195784	coding	149	305	ATCCGCTGGACCGCCATGTT	281
195785	coding	149	307	TCATCCGCTGGACCGCCATG	282
195786	coding	149	309	CTTCATCCGCTGGACCGCCA	283
195787	coding	149	313	CTCTCTTCATCCGCTGGACC	284
195788	coding	149	315	GCCTCTCTTCATCCGCTGGA	285
195789	coding	149	339	CCACATTGCCCAGGTCTCCA	286
195790	coding	149	341	AGCCACATTGCCCAGGTCTC	287
195791	coding	149	353	GTCCTTTCCAGCAGCCACAT	288
195792	coding	149	356	ACCGTCCTTTCCAGCAGCCA	289
195793	coding	149	371	GGACACATTGGCCACACCGT	290
195794	coding	149	377	TTCAATGGACACATTGGCCA	291
195795	coding	149	423	TACGGCCAATGATGGAATGC	292
195796	coding	149	437	GTGGACCACCATAGTACGGC	293
195797	coding	149	503	GCTTCCAGCATTTCCAGTCT	294
195798	coding	149	512	AGCCAAGCGGCTTCCAGCAT	295
195799	coding	149	518	ACCACAAGCCAAGCGGCTTC	296
195800	stop	149	537	ATTGGGCAATCCCAATCACA	297
195801	stop	149	541	GTTTATTGGGCAATCCCAAT	298
195802	stop	149	555	ACCACATAGGGAATGTTTAT	299
195803	stop	149	557	AGACCACATAGGGAATGTTT	300

195804	3'UTR	149	561	ACTCAGACCACATAGGGAAT	301
195805	3'UTR	149	565	TGAGACTCAGACCACATAGG	302
195806	3'UTR	149	571	TGAGTCTGAGACTCAGACCA	303
195807	3'UTR	149	573	GATGAGTCTGAGACTCAGAC	304
195808	3'UTR	149	575	CAGATGAGTCTGAGACTCAG	305
195809	3'UTR	149	577	AGCAGATGAGTCTGAGACTC	306
195810	3'UTR	149	581	GGACAGCAGATGAGTCTGAG	307
195811	3'UTR	149	588	TTTAGCAGGACAGCAGATGA	308
195812	3'UTR	149	590	AGTTTAGCAGGACAGCAGAT	309
195813	3'UTR	149	597	TTTCTACAGTTTAGCAGGAC	310
195814	3'UTR	149	599	TTTTTCTACAGTTTAGCAGG	311
195815	3'UTR	149	626	TTAAGATTACAGTTTAATGG	312
195816	3'UTR	149	628	TGTTAAGATTACAGTTTAAT	313
333624	coding	149	469	TTTCCACCTTTGCCCAAGTC	314

In addition to targeting rat superoxide dismutase 1, soluble mRNA, SEQ ID Nos 250 and 314 also target human superoxide dismutase 1, soluble.

5   In a further embodiment of the present invention, an oligonucleotide was designed to target the rat superoxide dismutase 1, soluble RNA, using published sequences (GenBank accession number Y00404.1, incorporated herein as SEQ ID NO: 149). This compound consists of the sequence

10   CTTCAGCACGCACACGGC (SEQ ID NO: 315). This compound is a chimeric oligonucleotide ("gapmers") 18 nucleotides in length, composed of a central "gap" region consisting of ten 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by four-nucleotide "wings". The wings

15   are composed of 2'-methoxyethyl (2'-MOE)nucleotides. The internucleoside (backbone) linkages are phosphorothioate (P=S) throughout the oligonucleotide. All cytidine residues are 5-methylcytidines. The ''target site'', which indicates the first (5'-most) nucleotide number on the

20   particular target sequence to which the oligonucleotide binds, is nucleotide 105 of SEQ ID NO: 149.

Example 21

Phosphorothioate oligodeoxynucleotides targeted to

25   **rat superoxide dismutase 1, soluble**

In a further embodiment of the present invention, a third series of oligonucleotides was designed to target

different regions of the rat superoxide dismutase 1,
soluble RNA, using published sequences (GenBank accession
number Y00404.1, incorporated herein as SEQ ID NO: 149;
GenBank accession number Z21924.1, incorporated herein as
5   SEQ ID NO: 155; GenBank accession number Z21920.1,
incorporated herein as SEQ ID NO: 154; GenBank accession
number NM_017050.1, incorporated herein as SEQ ID NO: 316;
genomic sequence representing nucleotides 5965000 to
5972000 of GenBank accession number NW_042743.1,
10 incorporated herein as SEQ ID NO: 317). The
oligonucleotides are shown in Table 7. ''Target site''
indicates the first (5'-most) nucleotide number on the
particular target sequence to which the oligonucleotide
binds. All compounds in Table 7 are oligodeoxynucleotides
15 20 nucleotides in length. The internucleoside (backbone)
linkages are phosphorothioate (P=S) throughout the
oligonucleotide. All cytidine residues are 5-
methylcytidines.

20   .   Table 7

Phosphorothioate oligodeoxynucleotides targeted to

rat superoxide dismutase 1, soluble

ISIS #	REGION	TARGET SEQ ID NO	TARGET SITE	SEQUENCE	SEQ ID NO
336649	coding	149	156	CACCGCTTGCCTTCTGCTCG	318
336651	coding	316	441	TCTCGTGGACCACCATAGTA	319
336653	intron	317	636	TGCAAAACGAGGGCCCAGCG	320
336655	exon:intron	317	802	GGGCCTTGCCTTCTGCTCGA	321
336657	intron:exon	317	2598	CACCGCTTGCCTTTATTTAA	322
336659	exon:intron	317	2696	TTAAGACTTACCTTGTGTAT	323
336661	intron:exon	317	4191	GTGGTACAGCCTATTTACCA	324
336663	exon:intron	317	4261	TGCTGCTCACCTCTCTTCAT	325
336665	intron:exon	317	4949	TCCAACATGCCTAACATTAA	326
336667	exon:intron	154	142	GGAAACTTACCACCATAGTA	327
336669	intron	154	154	TCTCGTGGACCTTTGAAAAG	328

In a further embodiment of the present invention, a
25 third series of oligonucleotides was designed to target
different regions of the rat superoxide dismutase 1,
soluble RNA, using published sequences (GenBank accession

number Y00404.1, incorporated herein as SEQ ID NO: 149;
GenBank accession number Z21920.1, incorporated herein as
SEQ ID NO: 154; GenBank accession number Z21924.1,
incorporated herein as SEQ ID NO: 155; GenBank accession
5   number NM_017050.1, incorporated herein as SEQ ID NO: 316;
genomic sequence representing nucleotides 5965000 to
5972000 of GenBank accession number NW_042743.1,
incorporated herein as SEQ ID NO: 317). The
oligonucleotides are shown in Table 8. ''Target site''
10   indicates the first (5'-most) nucleotide number on the
particular target sequence to which the oligonucleotide
binds. All compounds in Table 8 are oligodeoxynucleotides
15 nucleotides in length. The internucleoside (backbone)
linkages are phosphorothioate (P=S) throughout the
15   oligonucleotide. All cytidine residues are 5-
methylcytidines.

Table 8

Phosphorothioate oligodeoxynucleotides targeted to
20   rat superoxide dismutase 1, soluble

ISIS #	REGION	TARGET SEQ ID NO	TARGET SITE	SEQUENCE	SEQ ID NO
336650	coding	316	158	CGCTTGCCTTCTGCT	329
336652	coding	316	443	CGTGGACCACCATAG	330
336654	intron	317	638	AAAACGAGGGCCCAG	331
336656	exon:intron	317	804	CCTTGCCTTCTGCTC	332
336658	intron:exon	317	2600	CGCTTGCCTTTATTT	333
336660	exon:intron	317	2698	AGACTTACCTTGTGT	334
336662	intron:exon	317	4193	GTACAGCCTATTTAC	335
336664	exon:intron	317	4263	TGCTCACCTCTCTTC	336
336666	intron:exon	317	4951	AACATGCCTAACATT	337
336668	exon:intron	317	5069	AACTTACCACCATAG	338
336670	intron:exon	317	6017	CGTGGACCTTTGAAA	339

In addition to targeting rat superoxide dismutase 1,
soluble mRNA, SEQ ID NO: 130 also targets human superoxide
25   dismutase 1, soluble.

Example 22

Inhibition of superoxide dismutase 1, soluble mRNA in rat brain following intraventricular administration.

5   Oligonucleotides that target the expression of proteins in the brain represent a potential means of treating degenerative neurologic disorders associated with aberrant proteins such as superoxide dismutase 1, soluble that are associated with familial ALS. To be effective in
10   ALS, they would need to reach affected tissues.
       Superoxide dismutase 1, soluble mRNA levels were measured in rat brain following intraventricular administration. Superoxide dismutase 1, soluble levels were measured in both rat spinal cord and rat brain
15   following administration of ISIS 146192. Administration was performed daily at either 33 µg/day or 50 µg/day for 14 days. The results are shown in Figs. 1-4. There are two sets of bars for each dose since duplicate treatment groups of 6 per group were used. Expression is relative to PTEN
20   mRNA to demonstrate specificity. mRNA was normalized to ribogreen or beta-actin.
       The results show that intraventricular administration of ISIS 146192 significantly reduced superoxide dismutase 1, soluble mRNA levels in both the spinal cord and right
25   temporal parietal section of the brain. Thus, oligonucleotides are preferentially taken up by motor neurons in the brain stem and spinal cord, suggesting that cell barriers are not an obstacle. Thus, intraventricular administration of superoxide dismutase 1, soluble antisense
30   oligonucleotides has therapeutic implications in treatment of ALS and other neurodegenerative disorders.

Example 23

Design and screening of duplexed antisense compounds
targeting superoxide dismutase-1, soluble

In accordance with the present invention, a series of
5   nucleic acid duplexes comprising the antisense compounds of
the present invention and their complements can be designed
to target superoxide dismutase-1, soluble. The nucleobase
sequence of the antisense strand of the duplex comprises at
least a portion of an oligonucleotide to superoxide
10   dismutase-1, soluble as described herein. The ends of the
strands may be modified by the addition of one or more
natural or modified nucleobases to form an overhang. The
sense strand of the dsRNA is then designed and synthesized
as the complement of the antisense strand and may also
15   contain modifications or additions to either terminus. For
example, in one embodiment, both strands of the dsRNA
duplex would be complementary over the central nucleobases,
each having overhangs at one or both termini.   For
example, a duplex comprising an antisense strand having the
20   sequence CGAGAGGCGGACGGGACCG and having a two-nucleobase
overhang of deoxythymidine(dT) would have the following
structure:

```
            cgagaggcggacgggaccgTT      Antisense Strand
            |||||||||||||||||||
25          TTgctctccgcctgccctggc      Complement
```

RNA strands of the duplex can be synthesized by
methods disclosed herein or purchased from Dharmacon
Research Inc., (Lafayette, CO). Once synthesized, the
30   complementary strands are annealed. The single strands are
aliquoted and diluted to a concentration of 50 uM. Once
diluted, 30 uL of each strand is combined with 15uL of a 5X
solution of annealing buffer. The final concentration of
said buffer is 100 mM potassium acetate, 30 mM HEPES-KOH pH
35   7.4, and 2mM magnesium acetate. The final volume is 75 uL.
This solution is incubated for 1 minute at 90°C and then
centrifuged for 15 seconds. The tube is allowed to sit for
1 hour at 37°C at which time the dsRNA duplexes are used in

experimentation. The final concentration of the dsRNA duplex is 20 uM. This solution can be stored frozen (-20°C) and freeze-thawed up to 5 times.

5   Once prepared, the duplexed antisense compounds are evaluated for their ability to modulate superoxide dismutase-1, soluble expression according to the protocols described herein.

Example 26

10   Design of phenotypic assays and *in vivo* studies for the use of superoxide dismutase-1, soluble inhibitors

Phenotypic assays

Once superoxide dismutase-1, soluble inhibitors have

15   been identified by the methods disclosed herein, the compounds are further investigated in one or more phenotypic assays, each having measurable endpoints predictive of efficacy in the treatment of a particular disease state or condition. Phenotypic assays, kits and

20   reagents for their use are well known to those skilled in the art and are herein used to investigate the role and/or association superoxide dismutase-1, soluble in health and disease. Representative phenotypic assays, which can be purchased from any one of several commercial vendors,

25   include those for determining cell viability, cytotoxicity, proliferation or cell survival (Molecular Probes, Eugene, OR; PerkinElmer, Boston, MA), protein-based assays including enzymatic assays (Panvera, LLC, Madison, WI; BD Biosciences, Franklin Lakes, NJ; Oncogene Research

30   Products, San Diego, CA), cell regulation, signal transduction, inflammation, oxidative processes and apoptosis (Assay Designs Inc., Ann Arbor, MI), triglyceride accumulation (Sigma-Aldrich, St. Louis, MO), angiogenesis assays, tube formation assays, cytokine and hormone assays

35   and metabolic assays (Chemicon International Inc., Temecula, CA; Amersham Biosciences, Piscataway, NJ).

In one non-limiting example, cells determined to be appropriate for a particular phenotypic assay (i.e., MCF-7 cells selected for breast cancer studies; adipocytes for obesity studies) are treated with superoxide dismutase-1,

5   soluble inhibitors identified from the *in vitro* studies as well as control compounds at optimal concentrations which are determined by the methods described above. At the end of the treatment period, treated and untreated cells are analyzed by one or more methods specific for the assay to

10 determine phenotypic outcomes and endpoints.

Phenotypic endpoints include changes in cell morphology over time or treatment dose as well as changes in levels of cellular components such as proteins, lipids, nucleic acids, hormones, saccharides or metals.

15 Measurements of cellular status which include pH, stage of the cell cycle, intake or excretion of biological indicators by the cell, are also endpoints of interest. Analysis of the genotype of the cell (measurement of the expression of one or more of the genes of the cell) after

20 treatment is also used as an indicator of the efficacy or potency of superoxide dismutase-1, soluble inhibitors. Hallmark genes, or those genes suspected to be associated with a specific disease state, condition, or phenotype, are measured in both treated and untreated cells.

25